A POTENTIAL ROLE FOR EARLY GUT MICROBIAL COLONIZATION IN INFANT BEHAVIOR AND CHILD SLEEP DISORDERS

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ABSTRACT

A POTENTIAL ROLE FOR EARLY GUT MICROBIAL COLONIZATION IN INFANT BEHAVIOR AND CHILD SLEEP DISORDERS

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There is increasing evidence from pre-clinical and human studies implicating the microbiota–gut–brain axis in behavior and sleep physiology. Infancy is a critical time period for brain development and is vulnerable to the harmful effects of gut dysbiosis. Thus, it is crucial to understand how gut microbial colonization during this period may influence behavior and sleep physiology in the later stages of life.

We analyzed data from 194 mother-infant pairs from the Michigan Archive for Research on Child Health (MARCH) cohort Study. Clinical and demographic information was obtained from the birth certificate and interview during pregnancy and childhood. Fecal samples from infants at 3-9 months of age were sequenced at the V4 region of the 16S rRNA gene.

In the first study, which examined whether feeding practices may affect early gut microbial colonization, we found that the gut microbiota of infants who were exclusively breastfed displayed a significantly lower Shannon diversity (p-adjust < 0.001) and a different gut microbiota composition than infants who were not breastfed (p-value = 0.001). Among the exclusively breastfed infants, recipients of supplemental vitamin D displayed a significantly lower Shannon diversity (p-adjust = 0.007) and different gut microbiota composition structure than non-supplemented, breastfed infants (p-value = 0.02). In addition, several individual taxa were identified to be associated with different feeding practices.

In the second study, we examined whether gut microbiota in early infancy was associated with temperament in the nine-month-old infants. We identified that a microbial cluster characterized

by a higher abundance of *Bifidobacterium, Veillonella,* and *Escherichia-Shigella* that was associated with lower emotionality scores (coefficient = -0.58, p-value = 0.02) compared to a cluster characterized by a higher abundance of *Bacteroides*. This association was especially prominent among infants who were not supplemented with vitamin D (coefficient = -1.01, p-value = 0.01), while no significant association was found among infants who were supplemented (coefficient = -0.43, p-value = 0.20).

In the final aim, we assessed the association between gut microbiota in early infancy and the difficulty of initiating and maintaining sleep at age of two years. The gut microbiota of children who had difficulty maintaining sleep displayed significantly higher Shannon index (OR: 2.41, 95% CI= 1.23-4.93, p-adjust < 0.04) and Chao 1 index (OR: 1.01, 95% CI= 1.0-1.03, p-adjust < 0.008) after adjustment for covariates. We also observed that gut microbiota composition was significantly different between children with difficulty initiating (p-value= 0.043) and maintaining sleep (p-value= 0.004) by PERMANOVA based on the unweighted UniFrac distance metric.

In conclusion, these results from analysis in a prospective cohort study suggest that early gut microbial colonization is shaped by breastfeeding status, vitamin D supplement, and maternal characteristics including gestational age, delivery mode and education level. Our findings suggested that the infant gut microbiome clusters may be associated with the temperament characteristic of negative emotionality in 9-month-old infants. We also demonstrated a significant association between infant gut microbiome composition and sleep problems in 2-year-old children. Thus, our results add to the evidence that early gut microbial colonization may be linked with brain outcomes with potential long-term effects.

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KEY TO ABBREVIATIONS

MARCH	Michigan Archive for Research on Child Health
SCFAs	Short chain fatty acids
GF	Germ-free
CS	Caesarean section
HMOs	Human milk oligosaccharides
MGBA	Microbiota-gut-brain axis
CNS	Central nervous system
ANS	Autonomic nervous system
ENS	Enteric nervous system
HPA	Hypothalamic pituitary adrenal
SPF	Specific pathogen-free
ASD	Autism spectrum disorder
MTT	Microbiota transplantation therapy
OUT	Operational Taxonomic Unit
ASV	Amplicon Sequence Variant
PERMANOVA	Permutational multivariate analysis of variance
ECHO	Environmental Influences on Child Health Outcomes
NEG	Negative emotionality
PAS	Positive affect/surgency
ORC	Orienting and regulatory capacity
BMI	Body mass index

FDR	False discovery rate
MaAsLin	Multivariate association with linear models
ANOVA	Analysis of variance
PCoA	Principal coordinate analysis
VDR	Vitamin D receptor
IBQ-RVSF	Rothbart Infant Behavior Questionnaire-Revised Very Short Form
DMM	Dirichlet multinomial mixture
OSA	Obstructive sleep apnea
PSDS	PROMIS Sleep Disturbance scale
DSM-4	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition

CHAPTER1. INTRODUCTION

1.1 Background

The human gut microbiome is composed of a wide variety of microorganisms, including bacteria, archaea, viruses, and eukaryotic microbes that all reside in our intestines. Several basic functions conferred by the gut microbiome on the human host suggest its importance in health and disease. These functions include regulation of the immune system, protection against pathogens, and fermentation of indigestible food components into metabolites[1,2]. However, these functions can be disrupted by an altered microbial composition, which is referred to as dysbiosis[1]. Several diseases are now thought to be influenced by dysbiosis, including some types of cancer[3,4], mental disorders[5–8], inflammatory bowel diseases[9,10], type 2 diabetes[11,12], and obesity[1,13].

The gut microbiota produces a set of through the breakdown of indigestible carbohydrates [14,15]. Among the metabolites, short chain fatty acids (SCFAs) perform complex but important roles in the human body[16]. The three most prominent SCFAs are acetate, propionate, and butyrate, which occur in a ratio of approximately 3:1:1 in the human intestinal lumen, respectively[16]. Butyrate is thought to be the most important SCFA for human health, as it forms the major energy source for human colonocytes[17,18]. Butyrate also potentially prevents cancer activity by inducing apoptosis of colon cancer cells and by regulating gene expression by inhibiting histone deacetylases[19]. Propionate is also an energy source for the epithelial cells but is largely taken up by the liver[18]. The transformation of propionate to glucose in intestinal gluconeogenesis has beneficial effects on energy homeostasis by decreasing hepatic activity, and in turn, reduces adiposity[20]. Acetate is the most abundant SCFA, and is important for gut environment stability and the growth of beneficial bacteria[21]. In addition, Acetate can cross the

blood–brain barrier and regulate hypothalamic neuronal activation patterning and neuropeptide release[22].The joint study of the microbiome and the metabolome is considered one of the best approaches to study host–microbiome interactions[23,24].

The gut microbiota is also critical for the development of both the intestinal mucosal and systemic immune system as demonstrated by the studies in animal models. A number of studies have shown that germ-free (GF) mice are more susceptible to infection than specific-pathogen free mice[25]. In addition, administration of antibiotics is associated with an increased risk of pathogen colonization in both mice and humans, suggesting an important function of the commensal microbiota in protecting the host from infection[26,27]. A major immune deficiency in germ-free animals is the absence of expansion of CD4+ T-cell populations, which can be completely reversed by polysaccharide A produced by *B. fragili*[28,29]. This reversion is mainly performed by the pattern recognition receptors (PRRs) of epithelial cells, such as Toll-like or Nod-like receptors, which can recognize the molecular effectors that are produced by gut microbiota[30]. Dysbiosis can alter the microbiol molecules sensed by the host, and in turn lead to a different activation state of the immune system[31]. A recent research which studied prenatal and early life bacterial colonization found that by transferring altered gut microbiome from pregnant patients with Crohn's disease to germ-free mice, an imbalanced immune system lacking critical homeostatic elements was displayed in the GF mice, suggesting gut dysbiosis could trigger abnormal imprinting of the intestinal immune system[32].

1.2 Early microbial colonization

The colonization of gut bacteria begins at birth and remains highly dynamic until about 2-3 years of age when more stable microbial profiles begin to emerge[33,34]. Several reports highlighted that the early life development of the infant gut microbiota plays a critical role in the health of

later life, such as allergic diseases, obesity, and autism[33,35]. Mode of delivery, infant feeding practices, and antibiotic exposure are thought to be three key factors that influence early microbial colonization and establishment[36]. The relationship between these 3 factors and infant gut microbiome have been well established in the studies of recent years.

Mode of delivery is one of the major contributors to influencing the infant's gut microbiome. During vaginal delivery, the infant’s microbial colonization starts at the contact with the maternal vaginal and intestinal flora, while infants delivered by CS acquire bacteria derived from the maternal skin, mouth, and from the hospital environment[37,38]. The gut microbiota patterns of infants delivered by CS differ from those who were vaginally delivered, including lower diversity and richness, lower abundance of *Bacteroides, Lactobacillus*, and *Bifidobacterium*, and higher abundance of *Clostridium difficile* and microbes associated with the human skin such as *Staphylococcus, Streptococcus and Propionibacterium*[39–41]. Previous studies found that from birth to 90 days of life, *Bifidobacterium* and *Lactobacillus* were significantly lower in the infants delivered by CS compared with those delivered vaginally[42]. These differences in newborn gut microbiome community may have an impact on health since the genera *Bifidobacterium* and *Lactobacillus* are considered to be the beneficial bacteria in the gut[43].

Infant feeding practices are also key factors in shaping early microbiota composition[44,45]. Recent studies have shown that gut microbial profiles in breastfed infants are significantly different from those in formula-fed infants and change rapidly after the transition from breastfeeding to formula or solid food[46,47]. Specifically, breastfed infants have higher abundance of *bifidobacterial*, and lower microbial diversity compared with formula-fed infants whose gut microbiota is more diverse and similar to older children[47,48]. The differences in gut microbiota composition observed between formula-fed and breastfed infants have, at least

partially, been attributed to the absence of human milk oligosaccharides (HMOs) in infant formula[49]. Human milk is enriched with HMOs, which have been linked to beneficial bacteria in the gut microbiota[50,51]. In recent years, some oligosaccharides have been added to some formula to help infant to establish a Bifidobacterium-rich microbiota[51]. However, formula-fed infants still have distinct features of their microbiotas compared with breastfed infants, with a higher abundance of *C. difficile*[52]. The introduction of solid food represents another key factor of feeding practices influencing the composition of infant gut microbiota, producing an adult-like complex microbiome dominated by the phyla *Bacteroidetes* and *Firmicutes*[53,54]. Infant gut bacterial abundance changed significantly with the introduction of solid foods between 9 and 18 months. Specifically, the abundance of *Bacteroidetes* increases and the abundance of *Bifidobacterium* and *Lactobacillus* decrease[55,56].

During birth and immediately thereafter, broad-spectrum antibiotics are commonly used in newborns and their mothers who are at high risk for infection. However, antibiotics can also contribute to gut dysbiosis[57]. Early antibiotic exposure can reduce the diversity of the gut microbiota of infants and change its composition, with a decreased abundance of *Bifidobacterium* and increased abundance of *Proteobacteria*[58]. Healthy infants whose mothers received ampicillin for group B Streptococcus before delivery displayed significantly decreased abundance of *Bifidobacterium* by one week of life, highlighting the modulatory influences of intrapartum antibiotic interventions[59]. Studies have also demonstrated that the prophylactic antibiotic treatment in preterm infants can reduce the diversity of gut flora and delay the colonization of commensal flora[60,61].

1.3 The microbiota–gut–brain axis

The ‘microbiota-gut-brain axis’ (MGBA) refers to the biological network involving multiple

biological systems that allow bidirectional communication between gut bacteria and the brain[62]. This axis is becoming popular in fields investigating the biological and physiological basis of stroke, psychiatric, neurodevelopmental, age-related, and neurodegenerative disorders[62–64]. The communication network includes the central nervous system (CNS), both brain and spinal cord, the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary adrenal (HPA) axis[63]. The communication pathways in these biological networks include both direct and indirect signaling via neurotransmitters, metabolic pathways, and the immune system[62,63].

The most well-studied neuronal pathway for the MGBA is the vagus nerve signaling. The vagus nerve innervates the muscle and mucosa layers of the gut both in the lamina propria and muscularis externa, detects sensory signals and then relays these signals to the CNS[65,66]. The most important function of the vagus nerve is afferent activity which brings information of the inner organs, such as gut, liver, heart, and lungs to the brain[67]. A study reported that in vagotomized mice where the vagus nerve has been surgically severed, administration of *Lactobacillus rhamnosus* JB-1 does not affect GABA receptor expression[68]. whereas in normal mice, administration of *Lactobacillus rhamnosus* JB-1 alters the expression of GABA receptors in brain regions associated with fear and emotions.These receptors modulate anxiety-like behaviors, suggesting that the vagus as a major modulatory communication pathway between gut microbiota and the brain[68].

Inspired by how microbiota influence the brain through their ability to produce and modify many metabolic, immunological and neurochemical factors that impact the nervous system, flood of research is now connecting microbial communities, and their function to neuropsychiatric disorders associated with development (for example, autism spectrum disorder and

schizophrenia), with mood (for example, depression and anxiety) and with neurodegeneration (for example, Parkinson disease and Alzheimer disease)[69,70]. GF mice are one of the most widely used technical strategies in studying MGBA[71]. The main advantage of the GF mice model is that gut microbiome strains thought to be risk factors in gastrointestinal and systemic disease, such as candidate psychobiotics, can be studied in GF mice to learn more about their specific effects. In addition, fecal microbiota of human donors can be transplanted into GF mice to directly investigate the role of bacteria on disease pathogenesis. Several studies have shown that GF mice displayed increased stress response and decreased anxiety with augmented levels of adreno-corticotrophic hormone and cortisol compared with specific pathogen-free (SPF) mice with normal gut microbiota[72,73]. The abnormalities could be partially reversed when the gastrointestinal tracts of the germ-free mice were reconstituted with stool from normally raised mice[72]. The administration of microbiota from a patient with Alzheimer's disease has been shown to trigger cognitive decline in recipient GF mice[74]. In addition, metabolites related to the nervous system, including γ-aminobutyrate, taurine, and valine, were significantly less abundant in the feces of mice transplanted with microbiota from the affected patient, reinforcing the idea of a microbiota-gut-brain axis[74].

Human studies have also demonstrated that the gut microbiota is associated with neuropsychiatric and neurodegenerative disorders[70,75,76]. Autism spectrum disorder (ASD) are a serious neurodevelopmental disorder in children and about 1 in 44 children has been identified with ASD according to the estimates from CDC in the United States[77]. Children with ASDs have been found to have lower abundances of *Coprococcus, Prevotella,* and unclassified *Veillonellaceae,* and an increased Firmicutes/Bacteroidetes ratio[78,79]. Recent research has also reported beneficial effects of fecal microbiota transplantation therapy (MTT)

for individuals with ASD[80]. In this open-label clinical trial of MTT, 18 children diagnosed with ASD received an antibiotic treatment for 2 weeks followed by an initial, high dose of fecal microbiota transplants and subsequent lower maintenance doses administered daily for 7–8 weeks[80]. This study demonstrated that MTT appeared to reduce gastrointestinal symptoms (such as, constipation and abdominal pain) and improved ASD symptoms, such as social skills deficits and repetitive behavior. In a follow-up with the same participants after 2 years, most improvements in gastrointestinal symptoms were maintained, and autism-related symptoms improved even more after the end of treatment, suggesting a long-term impact[81]. The treatment also increased overall bacterial diversity and the abundance of *Bifidobacteria* and *Prevotella*, and these effects were remained over time as they were still observed in the 2-year follow-up[81]. The relationship between gut microbiota and cognitive development has been well studied in humans[82,83]. Carlson et al. demonstrated that higher alpha diversity of gut microbiota was associated with lower scores on the overall cognitive score, visual reception scale, and expressive language scale in 89 2-year-old children[82]. This study also showed that microbiome has minimal effects on regional brain volumes at 1 and 2 years of age by using MRI imaging[82].

1.4 Gut microbiome and sleep

Sleep quality and quantity are increasingly being considered as critical factors for child health and development. Epidemiology studies indicate that up to 50% of children experience a sleep disorder between 0 to 6 years old[84–86]. Difficulty initiating and maintaining sleep are the two most frequent sleep problems in childhood and often co-exist[87]. Spruyt et al. reported that 31% of children aged 6 to 13 years had disorder of initiating and maintaining sleep in a normal school-age population[88]. Liu et al. also showed that 16% of the parents in the United States

reported their children aged 4 to 11 years “sometimes” have difficulties falling asleep[89]. Multiple biological, psychosocial, and environmental factors are linked to sleep disorders in children[90]. Leone et al. found that gut microbiome dysbiosis could impair central and hepatic circadian clock gene expression, suggesting that gut microbiota may play a role in regulating or modifying circadian rhythm[91]. In a small group of breast cancer survivors (n=12), global sleep dysfunction was found to be associated with higher abundance of *Paracoccus*, *Rikenellaceae*, and *Clostridium*[92]. Another cross-sectional study of 37 healthy older adult found that global sleep dysfunction was associated with lower abundance of the phyla *Verrucomicrobia* and *Lentisphaerae*. Valentini et al. reported that children with Obstructive Sleep Apnea syndrome had a lower microbiota diversity and higher abundance of pro-inflammatory bacteria (*Proteobacteria, Clostridiaceae, Oscillospiraceae, Klebsiella*) compared to healthy participants[93].

1.5 Gut microbiome research methods

Nowadays, microbiome studies often rely on the analysis of 16S ribosomal RNA sequences for the taxonomic identification of bacteria. The 16S rRNA gene sequence is about 1,500 bp long and contains a highly conserved sequence that includes nine regions or windows of variable nucleotide sequence[94,95]. These nine regions constitute the most informative portions of the gene sequence for use in taxonomic classification. With the development of next-generation sequencing technology, 16S rRNA gene has become a powerful tool for pathogen detection and identification[94]. The pipeline for 16S amplicon analyses usually starts with using primers designed to amplify the hypervariable regions of the 16S rRNA gene (typically the V1–V3 region or the V3–V5 region). Sequences are clustered into Operational Taxonomic Units’ (OTUs) that contain similar 16S rRNA sequences with high sequence similarity[96]. A common

similarity threshold used is 97%, which was derived from an empirical study that showed most strains had 97% 16S rRNA sequence similarity[96]. For each OUT cluster, a single sequence is selected as a representative sequence, which is annotated using a 16S classification method[97]. The annotation is applied to all other sequences within the same OUT. Several pipelines have been developed to perform the entire 16S analysis from end to end, including QIIME and MOTHUR[98,99].

Recently, a new method called Amplicon Sequence Variant (ASV) approach has been developed. The ASV approach starts by determining which exact sequences are read and how many times each exact sequence is read from Illumina-scale amplicon data without imposing the arbitrary dissimilarity thresholds[100]. ASV methods infer the biological sequences in the sample before the introduction of amplification and sequencing errors. This allows ASV methods distinguish sequence variants differing from only one nucleotide[100]. Therefore, an ASV-based analysis is able to provide a higher-resolution taxonomic result allowing for more precise identification down to the species level and even potentially beyond[101].

Alpha diversity is the ecological diversity of a single sample and is commonly used as a measurable outcome in microbiome research with respect to its richness (number of different species present in an area), evenness (relative abundance of the different species in an area), or both[102]. In microbial ecology, analyzing the alpha diversity of gut microbiota data is a common first approach to assessing differences between environments. The Shannon index is one of the popular index for alpha diversity, which accounts for both richness and evenness in a single equation, while the Chao 1 index only accounts for the richness[103]. The diversity of gut microbiota within an individual has been linked to several human diseases. For example, low diversity in the gut has been associated with obesity and inflammatory bowel disease[104].

While alpha diversity is a measure of microbiome diversity in a single sample, beta diversity is a measure of similarity or dissimilarity between two communities. It provides a measure of the degree to which samples differ from one another and it can reveal the structure difference between microbiota samples[105]. Some of the most popular beta diversity measures in microbiome research include the Bray-Curtis index (compositional dissimilarity), the Jaccard index (presence / absence measures, ignoring abundance information), and the UniFrac distances (which take into account the phylogenetic tree information)[106]. Many popular statistical methods, such as ordination-based methods, and permutational multivariate analysis of variance (PERMANOVA) are relied on the beta diversity[107,108]. Beta diversity is widely used for studying the association between environmental variables and microbial composition.

1.6 Study population

The Michigan Archive for Research on Child Health (MARCH) cohort is an ongoing population-based pregnancy and birth cohort set in Michigan’s lower peninsula. Many important problems in child health and development may result from a mother's diet, her infections, and chemicals in her environment during her pregnancy. The MARCH study plans to examine 1,100 pregnancies in detail by interviewing women in pregnancy, acquiring abstracts of their medical records, and by saving biological specimens obtained in pregnancy (blood, urine, and placenta). The MARCH study assesses the child's health and development in relation to these factors, to learn what changes might be made during a woman's pregnancy that could prevent later problems in child health and development. The MARCH study contributes to a nation-wide study of child health called the Environmental Influences on Child Health Outcomes (ECHO). ECHO is a research program launched by the National Institutes of Health in 2016 to understand early environmental factors on child health and development from nearly 40 cohorts across the US. ECHO research

focuses on four key pediatric outcomes: pre-, peri-, and postnatal outcomes, upper and lower airway, obesity, and neurodevelopment. With the large sample size and generalizability of the study population, the ECHO program should allow for new insights into many prenatal factors and child health, that can lead to the development of interventions and prevention strategies to improve child health across the U.S[109].

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CHAPTER 2. SPECIFIC AIMS

The overall goal of this dissertation is to determine how feeding practices and other factors shape the early development of the infant gut microbiota, and to study the gut-microbiota-brain axis by assessing the relationship between gut microbiota and infant temperament at 9 months, and infant sleep problems at 2 years.

Aim 1: To determine the association between infant feeding practices and gut bacterial composition in early infancy, accounting for other factors affecting the gut microbiota.

Hypothesis1a: We hypothesize that the gut microbiota of infants who are exclusively breastfed will have higher abundance of *Bifidobacterial*, *Streptococcus*, and *Lactobacillus*, and a lower alpha diversity, compared with partially breastfed infants and exclusively formula infants at 3-9 months of infant life.

Hypothesis1b: We hypothesize that the composition of gut microbiota from partially breastfed shows a greater resemblance to the gut microbiota of exclusively breastfed infants than to the gut microbiota from exclusively formula infants.

Hypothesis1c: We hypothesize that among the exclusively breastfed, the gut microbiota of infants who had been given a vitamin D supplement will display a lower alpha diversity and a lower abundance of *Haemophilus.*

Aim 2: To determine whether gut microbiota at the age of 3 months is associated with infant temperament at the age of 9 months.

Hypothesis2a: We hypothesize that gut microbiome with higher alpha diversity at 3 months of age will be associated with higher scores of negative emotionality (NEG), and lower scores of positive affect/surgency (PAS) and orienting and regulatory capacity (ORC) of infant temperament at the age of 9 months.

Hypothesis2b: We hypothesize that a gut microbiome with higher abundance of beneficial bacteria, such as *Bifidobacterial*, *Streptococcus*, and *Lactobacillus* will be associated with lower scores of NEG, and higher scores of PAS and ORC of infant temperament at the age of 9 months.

Hypothesis2c: We hypothesize that gut microbiota clusters characterized by higher abundance of beneficial bacteria are associated with lower scores of NEG, and higher scores of PAS and ORC of infant temperament at the age of 9 months.

Aim 3: Determine whether gut microbiota in early infancy is associated with infant sleeping disorders at the age of 2 years.

Hypothesis3a: We hypothesize that a gut microbiome with higher alpha diversity in early infancy will be associated with a higher risk of difficulty initiating and maintaining sleep at the age of 2 years.

Hypothesis3b: We hypothesize that a gut microbiome with higher abundance of beneficial bacteria, such as *Bifidobacteria*, *Streptococcus*, and *Lactobacillus*, will be associated with a higher risk of difficulty initiating and maintaining sleep at the age of 2 years.

CHAPTER 3. VITAMIN D SUPPLEMENTATION IN EXCLUSIVELY BREASTFED INFANTS IS ASSOCIATED WITH ALTERATIONS IN THE FECAL MICROBIOME

3.1 Abstract

Breastfeeding and introduction of solid food are the two major components of infant feeding practices that influence gut microbiota composition in early infancy. But it is unclear whether additional factors influence the microbiota of infants either exclusively breastfed or not breastfed. We obtained 194 fecal samples from infants at 3-9 months of age, extracted DNA, and sequenced the V4 region of the 16S rRNA gene. Feeding practices and clinical information were collected by questionnaire and abstraction of birth certificates. The gut microbiota of infants who were exclusively breastfed displayed significantly lower Shannon diversity (p-adjust < 0.001) and different gut microbiota composition compared to infants who were not breastfed (p-value = 0.001). Among the exclusively breastfed infants, recipients of vitamin D supplements displayed significantly lower Shannon diversity (p-adjust = 0.007), and different gut microbiota composition structure than non-supplemented, breastfed infants (p-value = 0.02). MaAslin analysis identified microbial taxa that associated with breastfeeding and vitamin D supplementation. Breastfeeding and infant vitamin D supplement intake play an important role in shaping infant gut microbiota.

3.2 Introduction

The gut microbiota has been considered an "invisible organ" of the human body, playing important roles in modulating host functions, including metabolism, digestion, and gut mucosal immune responses and integrity[1,2]. Dysbiosis of the gut microbiota may be associated with various adverse health outcomes in infants such as asthma, Crohn's disease, inflammatory bowel disease, and type 1 diabetes (T1D)[3–7]. The colonization of gut bacteria begins at birth and remains remarkably dynamic until about 2-3 years of age when more stable microbial profiles

begin to emerge[8,9]. In addition to the mode of delivery and antibiotic exposure, infant feeding practices are key factors in shaping early microbiota composition[10,11]. Recent studies have shown that gut microbial profiles in breastfed infants are significantly different from those in formula-fed infants and change rapidly after the transition from breastfeeding to formula or solid food[12–15]. The differences in gut microbiota composition observed between formula-fed and breastfed infants have, at least partially, been attributed to the absence of human milk oligosaccharides (HMOs) in infant formula[16]. Human milk is enriched with HMOs, which have been linked to beneficial bacteria in the gut microbiota[17,18]. The introduction of solid food represents another key factor influencing the composition of infant gut microbiota, producing an adult-type complex microbiome dominated by the phyla Bacteroidetes and Firmicutes[19,20].

Most gut microbiota research on infant nutrition to date has focused on breastfeeding and the introduction of solid food. Little is known about the effect of other dietary features within the two different feeding practices. It is recommended that babies who are breastfed exclusively should take vitamin D supplements every day due to the variability of vitamin D content in human breastmilk[21]. Because all infant formula in the United States is fortified with vitamin D, infants who are fed exclusively with formula usually do not need a vitamin D supplementation. Vitamin D not only prevents rickets, but also plays an important role in immune responses and metabolic processes that maintain the integrity of the gut epithelium[22–24].

Human milk can also be provided by bottle, from banking of milk by the mother or from human milk banks. This indirect form of breastfeeding can lead to enrichment by environmental bacteria, such as *Stenotrophomonas* and *Pseudomonadacea*[25]. The water used to reconstitute

powdered infant formulas may also be an important exposure for infant health outcomes. Reconstituting formula with tap water can lead to excessive fluoride and lead intake [26,27]. Different water types (e.g., city water, well water, filtration systems) can be a source of varied bacterial composition.

We sought to determine the association between maternal and infant characteristics and infant feeding practices and the gut microbiota profiles in 3- to 9-month-old infants. In addition, we analyzed the exclusively breastfed and non-breastfed infants separately to assess the impact of unique feeding practices features on the microbiotas of the infants in each group.

3.3 Materials and Methods

3.3.1 Study participants

The study population was drawn from the Michigan Archive for Research on Child Health (MARCH) cohort[28], an ongoing population-based pregnancy and birth cohort set in Michigan's lower peninsula. The purpose of the MARCH study is to store biological specimens and other health information in pregnancy and early life that can be used to better understand the causes of problems in pregnancy and optimize the health of children. This cohort is a component of a nation-wide study of child health called the Environmental influences on Child Health Outcomes (ECHO)[29]. Our analysis included mothers who provided informed consent for providing infant stool samples. During the MARCH 3 month phone interview mothers confirmed their interest in participating in this sample collection. Fecal collection kits were sent by mail. 194 fecal samples have so far been collected from singleton infants aged from 3 to 9 months old. The infants in this analysis were 3-9 months of age between 2018 and 2021.

3.3.2 Data collection

Several questionnaires were administered to mothers from the first prenatal visit through 9 months postpartum. The questionnaire at the first prenatal visit included demographic information about the mothers, their breastfeeding plans and many health-related practices and conditions as well as their estimated due date. Infant dietary feeding patterns, including breast milk and/or formula, detailed feeding practices, and complementary food intake, were collected at the same time as the fecal samples. Detailed information, including the infant's sex, birth weight, complications of pregnancy, mode of delivery (vaginal vs C-section), pre-pregnancy BMI and gestational age, was abstracted from the birth certificate.

3.3.3 Fecal microbiota analysis

Once received in the lab, the fecal samples were aliquoted into sterile tubes and stored at -80°C. DNA was extracted following a modified version of the Human Microbiome Project's protocol as described previously [30]. Barcoded primers were used to amplify the V4 region of the 16S rRNA gene following the mothur wet lab documentation. PCR amplification also followed the wet lab protocol outlined in the mothur documentation. The resulting 16S rRNA libraries were sequenced using 250 base pair Illumina MiSeq with V2 chemistry at the Michigan State University genomics core. After trimming, clean sequences were analyzed using the QIIME2 (2021.2 version) pipeline[31]. Demultiplexed sequences were further quality filtered and clustered using QIIME2's DADA2 plugin to generate the ASV table[32]. Unique amplicon sequence variants (ASVs) were assigned a taxonomy by the QIIME2 feature-classifier plugin, using the Silva 132 database at the similarity threshold of 99% (for 16S data)[33,34]. Samples were rarefied to 6,000 sequencing reads per sample, leaving 191 stool samples with 6,905 unique ASVs, and findings were summarized at the genus taxonomic level.

3.3.4 Statistical analysis

We used multivariate ordinal logistic regression models to estimate the association between pre-pregnancy BMI and breastfeeding practices, with adjustment for demographic variables and delivery mode.

Gut microbiota is analyzed by alpha diversity (Chao1 and Shannon) and beta diversity (Bray–Curtis dissimilarity and Weighted UniFrac) using the "vegan" package in R[35]. The difference of alpha diversity and relative abundance of taxa between feeding practices groups were tested by Wilcoxon rank test and Kruskal–Wallis test with false discovery rate (FDR) correction for multiple comparisons. We assessed the influence of factors significantly associated with gut bacterial community structure by multivariate models using a Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations based on Bray-Curtis dissimilarities (adonis, R vegan package)[35,36]. PERMANOVA is non-parametric multivariate statistical test, with p-values obtained using appropriate distribution-free permutation techniques. We used the multivariate association with linear models (MaAsLin) to identify associated microbiological taxa with the feeding practices and other related factors[37,38]. MaAsLin is a multivariate statistical framework that identifies associations between clinical metadata and microbial community abundance and provides both nominal p-values and FDR adjusted p-values (q-values) by Benjamini–Hochberg procedure[39]. Associations are considered significant when the q-value is below the threshold of 0.1.

3.4 Results

3.4.1 Participants and feeding practices

We analyzed gut microbiome samples from 191 infants. The distribution of the age at stool collection was shown in Figure 3.1. In Table 3.1, maternal and infant characteristics are compared by breastfeeding status (exclusive breastfeeding, partial breastfeeding, and not

breastfeeding). During the week immediately preceding stool sample collection, 88 (46.1%) infants were fed exclusively with breast milk, 43 (22.5%) were fed partially with breast milk, and 60 (31.4%) were not fed with breast milk. The median age at the time of specimen collection was 3.8 months (range: 3.0 months – 9.3 months). Partially breastfed infants were more likely to be fed with complementary foods than those who were not breastfed (44.2% vs 35.0%, $P > 0.4$). Infants who were exclusively breastfed were more likely to be given vitamin D supplementation than partially breastfed or not-breastfed infants (39.8% vs 18.6% vs 1.7%, $P<0.01$). A higher proportion of mothers who practiced exclusive breastfeeding were of normal BMI (18.5- 25.0) prior to pregnancy comparing to those who practiced partial breastfeeding or who were not breastfeeding (50.0% vs 39.5% vs 26.7%, $P<0.01$). Mothers who practiced exclusive breastfeeding were more likely to have a college degree than women who partially breastfed or did not breastfeed (72.4% vs 61.9% vs 32.2%, $P<0.01$). In a multivariate model adjusted for maternal age, maternal educational level, pre-pregnancy BMI (continuous), delivery mode, and infant age, mothers with higher pre-pregnancy BMI were less likely to practice breastfeeding (OR = 0.95, CI:0.91-0.99, p-value = 0.01, Table 3.2), and mothers with higher educational level were more likely to practice breastfeeding (OR= 2.66, CI: 1.72 - 4.21, p-value < 0.001, Table 3.2).

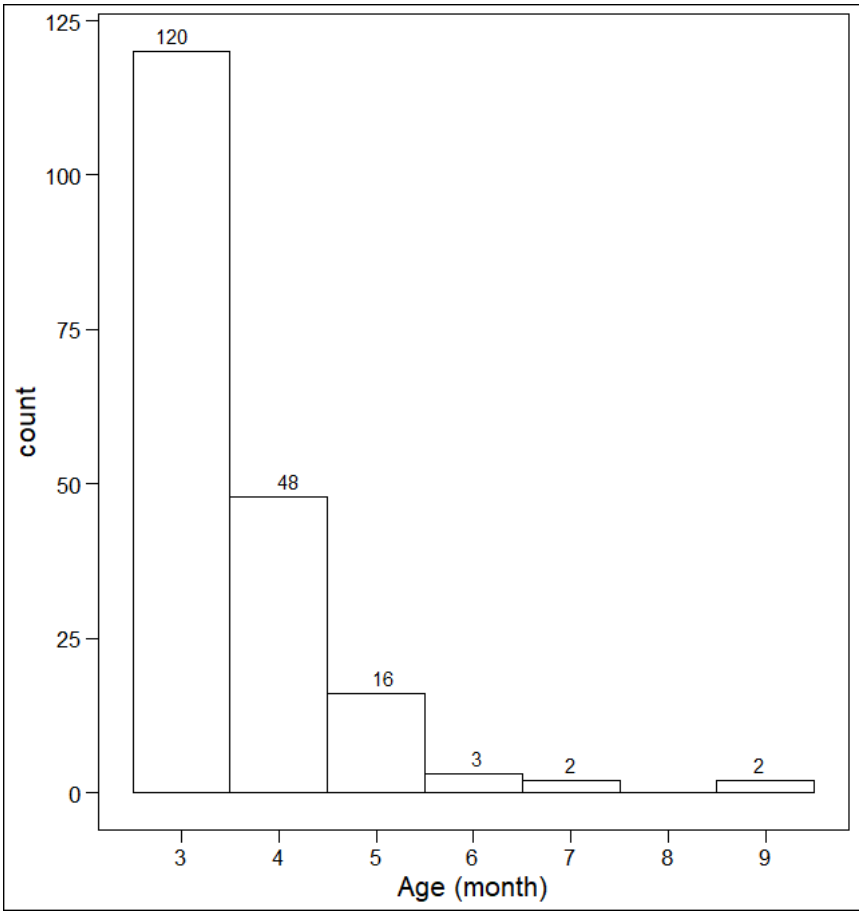

Figure 3.1. Distribution of infant age (month) at stool sample collection

Table 3.1. Characteristics of the mothers and infants by breastfeeding status[1]

Characteristic	Exclusive breastfeeding (N=88)	Partial breastfeeding (N=43)	No breastfeeding (N=60)	P-value
Infant age at sample collection (day), mean (SD)	115.5(17.3)	135.9 (39.8)	126.3 (31.9)	<0.01
Baby had any antibiotics since birth, n (%)	14 (15.9)	5 (11.6)	8 (13.3)	0.40
Consumption of complementary food during past 24 hours, n (%)	0 (0.0)	19 (44.2)	21 (35.0)	<0.01[3]
Infant probiotic supplement[2] during past 24 hours, n (%)	4 (4.5)	1 (2.4)	3 (3.3)	0.90
Infant Vitamin D supplement during past 24 hours, n (%)	35 (39.8)	8 (18.6)	1 (1.7)	<0.01
Delivery mode, n (%)				
Vaginal delivery	66 (75)	30 (69.8)	36 (60)	0.10
C-section	22 (25)	13 (30.2)	24 (40)	
Baby weight at delivery (gram), mean (SD)	3461 (551)	3336 (529)	3269 (598)	0.10
Baby sex, n (%)				
Male	43 (48.9)	22 (51.2)	30 (50)	0.98
Female	45 (51.1)	21 (48.8)	30 (50)	
Maternal pre-pregnancy BMI, n (%)				
<18.5	1 (1.1)	0 (0.0)	3 (5.0)	<0.01
18.5-25	44 (50)	17 (39.5)	16 (26.7)	
>25-30	24 (27.3)	11 (25.6)	12 (20.0)	
>30	19 (21.6)	15 (34.9)	29 (48.3)	
Maternal education level, n (%)				
Did not finish high school	0 (0.0)	0 (0.0)	6 (10.2)	<0.01
High school graduate or GED	4 (4.6)	4 (9.5)	21 (35.6)	
Some college	20 (23.0)	12 (28.6)	13 (22.0)	
College graduate or more	63 (72.4)	26 (61.9)	19 (32.2)	

[1]Breastfeeding status information was collected at the time of fecal sample collection. Values are mean (SD) for continuous variables or n (%) for categorical variables. Difference by breastfeeding status was calculated using an ANOVA or chi-squared test.
[2]Including probiotic supplement, kefir and kimchi
[3]Post hoc analysis with Bonferroni adjustment showed significant difference in consumption of complementary food between partial breastfeeding and no breastfeeding groups.

Table 3.2. Association between breastfeeding and perinatal characteristics[1]

Maternal characteristics	Proportional odds ratio	95% CI	p-value
Maternal age(year)	1.02	0.96-1.09	0.48
Maternal educational level	2.66	1.72-4.21	<0.001
Pre-pregnancy BMI (continuous)	0.95	0.91-0.99	0.01
Delivery mode (C-section vs Vaginal)	0.56	0.29-1.05	0.07
Infant age (day)	0.99	0.98-1.0	0.07

[1]A multivariate ordinal logistic regression analysis was performed to assess the association. Variables in the model include maternal age, maternal education level, pre-pregnancy BMI, delivery mode, and infant age.

3.4.2 Gut microbiota analysis

Fecal samples from infants who were exclusively breastfed displayed lower Shannon diversity than samples from those who were not breastfed (FDR adjusted p-value <0.01, Figure 3.2A). Samples from infants who were partially breastfed displayed Shannon diversity intermediate between the other two groups, but not significantly different from either. (FDR adjusted p-value = 0.9). Chao 1 index was not significantly different across the three groups (FDR adjusted p-value =1.0, Figure 3.2B). Among the exclusively breastfed, infants who had been given a vitamin D supplement during the previous 24 hours displayed lower Shannon index (FDR adjusted p-value < 0.01, Figure 3.2C) and lower Chao 1 index (FDR adjusted p-value = 0.6, Figure 3.2D) than those infants who were not given a vitamin D supplement. The multivariate model confirmed that fecal samples from infants who were not breastfed displayed significantly higher Shannon diversity than samples from those who were exclusively breastfed (Beta=0.18,

95% CI: 0.01, 0.34, p-value= 0.03, Table S3.1). The multivariate model also confirmed that fecal samples from infants who had been given a vitamin D supplement during the previous 24 hours displayed significantly lower Shannon index than those who were not given a vitamin D supplement (Beta=-0.27, 95% CI=-0.48, -0.08, p-value= 0.006, Table S3.2) in exclusively breastfed infants. All the multivariate models were adjusted for maternal age, maternal educational level, pre-pregnancy BMI (continuous), delivery mode, and infant age.

Whether breast milk was fed directly or was pumped and fed to the infant using a bottle, the Shannon and Chao 1 indices of the infant gut microbiota alpha diversity were similar (FDR adjusted p-value =1.0 and 0.88, respectively, Figure S3.1). Among the non-breastfed infants, neither the water type used to reconstitute the formula nor the consumption of complementary food during past 24 hours was associated with gut microbiota alpha diversity as measured by the Shannon or Chao 1 indices (Figure S3.1).

When classified by breastfeeding status, the gut microbiota communities of the infants were well separated in principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix (univariate PERMANOVA: R^2= 4.1%, p-value <0.01, Figure 3.2E). In addition to the feeding practices, gestational age (R^2=4.0%, p-value = 0.001) and delivery mode (R^2=2.0%, p-value = 0.003) were significantly associated with overall gut microbiome composition (Table 3.3; multivariate PERMANOVA model on Bray-Curtis distances). The PERMANOVA results were consistent with results from the Weighted UniFrac distance metric (Table S3.3). We then repeated the PERMANOVA analysis on Bray-Curtis distances within exclusively breastfed and not breastfed infants separately. These subgroup analyses also included additional variables. Accordingly, delivery mode (R^2 = 3.5%, P = 0.01) and infant vitamin D supplement in the past 24 hours (R^2 = 3.4%, P = 0.02) were significantly associated with gut microbiota composition in

exclusively breastfed infants (Table S3.4). Among the not breastfed infants, only maternal education level (R^2 = 4.1%, p-value = 0.02) was significantly associated with gut microbiota composition (Table S3.5). The PERMANOVA results of these two subgroup analyses were consistent with results from the Weighted UniFrac distance metrics (results not shown).

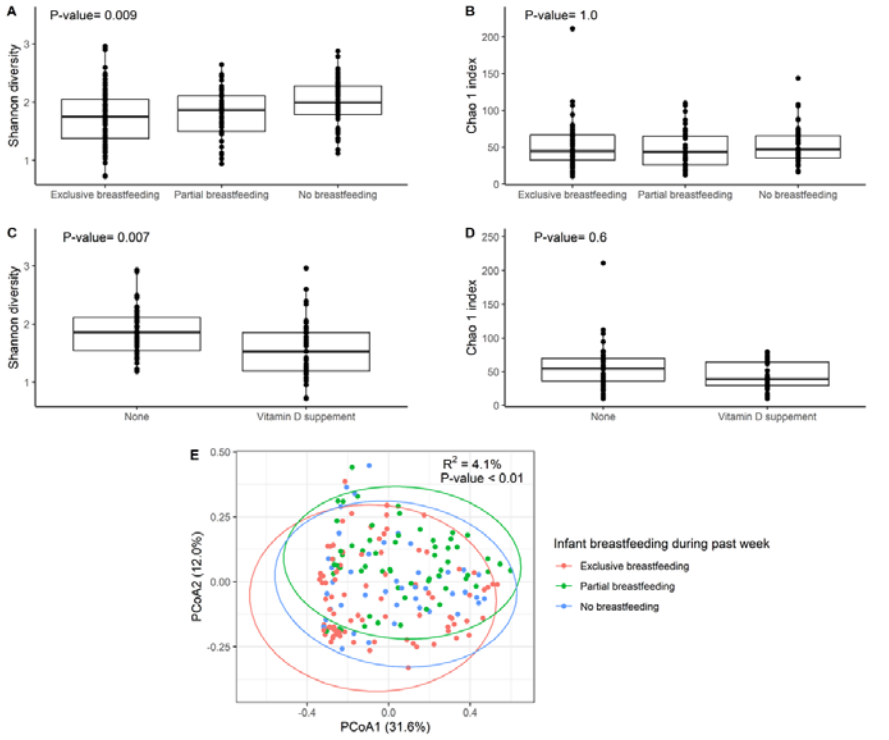

Figure 3.2. Infant alpha and beta diversity by infant breastfeeding and Vitamin D supplement. FDR adjusted p-value for alpha diversity was displayed in upper-left. (A) The Shannon diversity was used for alpha diversity. All the participants were included in the analysis (N=191). Group differences were tested by Kruskal-Wallis test. We then performed post hoc test for multiple comparisons. After FDR adjustment, no breastfeeding group has significant difference with exclusive breastfeeding (adjusted p-value <0.01). Partial breastfeeding group has no significant difference with exclusive breastfeeding group (p-value= 0.4, adjusted p-value=0.9) and no breastfeeding group (p-value= 0.03, adjusted p-value=0.09). (B) The Chao 1 index was used for alpha diversity. All the participants were included in the analysis (N=191). Post hoc test didn't find any significant difference between groups. (C) The Shannon diversity was used for alpha diversity. Only breastfeeding participants were included in this subgroup (N=92). Group differences were tested by Wilcoxon rank test. (D) The Chao 1 index was used for alpha diversity. Only breastfeeding participants were included in this subgroup analysis (N=92). (E) Principal component analysis (PCoA) ordinations of variation based on the Bray–Curtis distance matrix for all infants. R^2 and p-value were calculated by univariate PERMANOVA test.

Table 3.3. Results of Permutational Multivariate Analysis of Variance (PERMANOVA) for all infants

Variable	F value	R^2	p-value
Breastfeeding during past week	4	4.10%	0.001*
Gestational age	2.3	1.20%	0.03*
Infant sex	1.1	0.50%	0.36
Delivery mode (vaginal vs C-section)	3.6	1.80%	0.004*
Baby weight at delivery	0.7	0.30%	0.72
Infant probiotic supplement during past 24 hours	1	0.50%	0.38
Infant had any antibiotics since birth	0.9	1.00%	0.47
Maternal educational level	2.7	1.30%	0.02*
Maternal pre-pregnancy BMI (continuous)	0.37	0.20%	0.96

[1]Bray-Curtis distance was used for the PERMANOVA
* indicates the p-value <0.05

We further assessed the association between breastfeeding status and relative abundance of 8 dominant genera by univariate analysis (Figure S3.2). These 8 dominant genera were *Bacteroides, Bifidobacterium, Veillonella, Escherichia-Shigella, Ruminococcus gnavus, Clostridium sensu stricto 1, Prevotella,* and *Lachnoclostridium*. Exclusive breastfeeding was significantly associated with a higher relative abundance of *Bifidobacterium* (FDR adjusted p-value= 5×10^{-5}) and a lower relative abundance of *Lachnoclostridium* (FDR adjusted p-value $=5.6 \times 10^{-7}$).

MaAsLin results revealed that exclusive breastfeeding was significantly associated with the relative abundance of a set of genera, including *Intestinibacter, Flavonifractor, Lachnoclostridium, Clostridium innocuum group, Lactobacillus, Bifidobacterium* etc. (Table 3.4). Infant age at sample collection and maternal pre-pregnancy BMI were associated with higher relative abundance of *Lachnospira* and *Alistipes*, respectively (Table 3.4). Among exclusively breastfed infants, infants who had taken a vitamin D supplement in the previous 24 hours had a lower relative abundance of *Haemophilus* (Table 3.5). Among the not breastfed infants, having taken a probiotic supplement in the past 24 hours was associated with higher relative abundance of uncultured *Lachnospiraceae* and *Faecalitalea* (Table 3.5). Maternal pre-pregnancy BMI was associated with a higher relative abundance of *Alistipes (*Table 3.5).

Table 3.4. MaAsLin Analysis Results: Associations of infant feeding practices and gut microbiome taxa at genus level adjusted by covariates in all infants[1]

Taxonomy at genus level	Meta data value	Coefficient	N/N not 0	p-value	q-value[2]
Intestinibacter	Exclusive breastfeeding	-0.567	191/64	3.6×10^{-10}	3.0×10^{-7}
Flavonifractor	Exclusive breastfeeding	-0.896	191/145	4.0×10^{-8}	1.7×10^{-5}
Lachnoclostridium	Exclusive breastfeeding	-0.998	191/146	1.9×10^{-7}	5.2×10^{-5}
Clostridium innocuum group	Exclusive breastfeeding	-0.393	19144	4.9×10^{-6}	6.8×10^{-4}
Lactobacillus	Exclusive breastfeeding	0.68	191/115	4.3×10^{-6}	6.8×10^{-4}
Lactococcus	Exclusive breastfeeding	-0.287	191/29	1.7×10^{-5}	1.7×10^{-3}
Bifidobacterium	Exclusive breastfeeding	0.535	191/186	2.4×10^{-4}	0.018
Eisenbergiella	Exclusive breastfeeding	-0.322	191/24	2.4×10^{-4}	0.018
Colidextribacter	Exclusive breastfeeding	-0.398	191/40	3.2×10^{-4}	0.022
Akkermansia	Exclusive breastfeeding	-0.55	191/124	1.3×10^{-3}	0.066
Uncultured *Lachnospiraceae*	Exclusive breastfeeding	-0.188	191/20	1.4×10^{-3}	0.069

Table 3.4. (cont'd)

Haemophilus	Exclusive breastfeeding	0.463	191/141	1.7×10^{-3}	0.073
Staphylococcus	Exclusive breastfeeding	0.293	191/56	1.8×10^{-3}	0.073
Incertae_Sedis	Exclusive breastfeeding	-0.358	191/99	1.6×10^{-3}	0.073
Flavonifractor	Partial breastfeeding	-0.909	191/145	7.2×10^{-7}	1.5×10^{-4}
Haemophilus	Partial breastfeeding	0.74	191/141	1.3×10^{-5}	0.001
Lachnoclostridium	Partial breastfeeding	-0.86	191/146	5.8×10^{-5}	0.005
Lactococcus	Partial breastfeeding	-0.258	191/29	5.6×10^{-4}	0.031
Alistipes	Pre-pregnancy BMI	0.206	191/99	4.0×10^{-4}	0.025
Lachnospira	Age at sample collection	0.171	191/84	5.7×10^{-4}	0.032

[1]Model was adjusted for infant antibiotic use, sex, infant birth weight, delivery mode, age at fecal sample collection, infant probiotic supplement and pre-pregnancy BMI. Not breastfeeding is the reference for the breastfeeding status in the regression model.
[2]q-value is the FDR (Benjamini-Hochberg) adjusted p-value. q-value < 0.1 for multiple comparisons was considered statistically significant and included in the table.

Table 3.5. MaAsLin Analysis Results: Associations of infant feeding practices and gut microbiome taxa at genus level within exclusively breastfed and no breastfed infants[1]

Breastfeeding status	Taxonomy at genus level	Meta data value	Coefficient	N/N not 0	p-value	q-value[2]
Exclusively	*Haemophilus*	Vitamin D supplement (Yes)	-0.683	88/74	6.7×10^{-5}	0.058
	Faecalitalea	Probiotic supplement (Yes)	1.718	60/9	2.9×10^{-9}	2.4×10^{-6}
Not breastfeeding	Uncultured *Lachnospiraceae*	Probiotic supplement (Yes)	1.269	60/10	5.1×10^{-5}	0.021
	Alistipes	Pre-pregnancy BMI	0.431	60/27	2.6×10^{-4}	0.072

[1]Both Models were adjusted for infant antibiotic use, sex, infant birth weight, delivery mode, age at fecal sample collection, infant probiotic supplement and pre-pregnancy BMI.
[2]q-value is the FDR (Benjamini-Hochberg) adjusted p-value. q-value < 0.1 for multiple comparisons was considered statistically significant and results were included in the table.

3.5 Discussion

Our study was conducted in a population with somewhat higher than average rates of exclusive breastfeeding, with nearly half (46.1%) of the infants exclusively breastfed at the median age of 3.8 months. This percentage is higher than found in the Infant Feeding Practices Study II in the US (34% at 3 months) in 2007 [40], while it is closed to the percentage in the CDC National Immunization Survey in 2018 (46.3% at 3 month)[41]. Although the American Academy of Pediatrics recommends vitamin D supplementation for all breast-fed babies[42], only 39.8% of the exclusively breastfed infants in our study followed this recommendation, a lower frequency than that has been found in Canadian and European cohorts in 2009 and 2014, respectively[43,44].

We found that higher maternal education level and lower pre-pregnancy BMI were independently and significantly associated with an increased odds of being exclusively breastfed, consistent with previous studies in developed and developing countries[40,45,46]. Breastfeeding initiation and duration are also negatively correlated with high pre-pregnancy BMI[47,48]. These associations may be attributed to the physiological factors such as delayed onset of lactogenesis II and imbalances of hormones[49]. Previous studies have showed that maternal obesity can cause the delayed onset of lactogenesis II (DOL), a hormonal process that is associated with mother's confidence that her milk is sufficient for her child[50,51]. As a result, it can lead to lower rates of breastfeeding initiation and early termination of exclusive breastfeeding. The associations between maternal BMI and lactation success have been recently reviewed[52,53]. Our study demonstrated the importance of both breastfeeding and infant vitamin D supplements in shaping infant gut microbiota composition. Breastfeeding is significantly associated with both alpha and beta diversity of infant gut microbiota. We observed that the Shannon diversity of partially breastfed infants was between that of exclusively breastfed infants and not breastfed infants, though somewhat closer to the exclusively breast fed, suggesting a dose-response relationship between breastfeeding and Shannon diversity of infant gut microbiota. These results agree with a previous study, which reported that the composition of gut microbiota from partially breastfed infants are similar to that from exclusively breastfed infants[13]. Among the subgroup analysis of exclusively breastfed infants, the alpha and beta diversity results demonstrated that vitamin D supplementation is associated with infant gut microbiota overall composition. These results are in good agreement with the study of Lei et al. who investigated the role of vitamin D supplement on gut microbiome from 31 exclusively breastfed infants at 4-months-old[54]. Animal studies demonstrate that vitamin D plays a critical role in maintaining the integrity of the

intestinal mucosal barrier by preserving the integrity of junctions that control mucosal permeability and reduction of pro-inflammatory cytokines such as IL-8[55–57]. In addition, studies also found that VDR-mediated signaling inhibits inflammation-induced apoptosis of intestinal epithelial cells[56,58]. As a result of these effects on the intestinal mucosa, vitamin D acts as an important factor influencing the gut microbiota.

Besides the infant feeding practices, the results herein confirm that gestational age, delivery mode and maternal educational level are also significantly associated with gut microbiome composition. These results are consistent with many previous studies[10,13,59]. However, delivery mode is only associated with gut microbiota composition among the exclusively breastfed infants, while no significant association was found in the not breastfed infants. This might be attributed to the fact that c-section delivery can delay lactation initiation[60] and shape the bacterial composition of breast milk[61,62]. Maternal educational level is the only factor that significantly associated with infant gut microbiota composition among the not breastfed infants, whereas it was not significant among the exclusively breastfed infants. This finding suggests that not breastfed infants are more susceptible to socio-economic factors, such as educational level, which is normally be connected to offspring diet and nutritional status[63]. Hence, this association among the not breastfed infants might be mediated by the types of solid food introduction and quality of formula purchased. However, our data set did not allow us to test these associations.

Our study confirmed that *Bifidobacterium* was enriched in breastfed infants when compared with non-breastfed infants. Lower abundance of *Bifidobacterium* in infants due to early cessation of breastfeeding could potentially inhibit the interaction of bifidobacterial-mediated metabolites with the immune system, leading to higher levels of inflammation[64,65]. In contrast, the genus

Lachnoclostridium (*Lachnospiraceae* family) was found to be enriched in the non-breastfed infants when compared with exclusively breastfed or partially breastfed infants. In addition, the genera *Eisenbergiella* and *Lachnospiraceae*_uncultured, which also belong to *Lachnospiraceae* family were found to be enriched in the non-breastfed infants by MaAslin. These observations agree with previous studies that lower abundance of *Lachnospiraceae* is associated with breastfeeding at 3 months of age[66]. The evidence from many studies showed that *Lachnospiraceae* family or specific genera of *Lachnospiraceae* may be associated with several inflammatory conditions, such as metabolic syndrome, obesity, diabetes, and liver diseases[67–70].

Notably, the genus *Haemophilus* was enriched in the breastfed infants. However, exclusively breastfed infants who had taken a vitamin D supplement in the past 24 hours had a lower relative abundance of *Haemophilus* compared to those exclusively breastfed infants who were not supplemented. Consistent with our study, Fehr et al. showed that breastmilk may specifically provide *Haemophilus* to the infant gut[13]. Luthold et al also demonstrated that *Haemophilus* was less abundant in the group of highest vitamin D intake[71], supporting the hypothesis that a reduced immune response in vitamin D deficiency could augment the competitive advantage of *Haemophilus* and influence the composition of the infant gut microbiome[72].

Our study did not detect any effect of feeding with expressed milk, infant antibiotic intake, and water type for formula on gut microbiome. However, sample sizes were small for many of these comparisons. Therefore, pooling data from multiple cohort studies or analysis in larger cohorts with similar data are necessary to confirm this lack of association. For instance, previous studies demonstrated that infants who had been exposed to antibiotics had decreased abundance of *Bifidobacteria* and *Bacteroides* in the infant gut microbiome[73]. In our study, we asked the

mothers if the infant had taken any antibiotics since birth, whereas the timing of antibiotics administration was unknown. Hence, it's possible that infant gut microbiome had recovered from the dysbiosis states caused by antibiotics at the time of stool sample collection. The inconsistent results may also be attributed to variations in the antibiotic type, dosage, duration[74].

An important limitation of this study is that only a single stool sample was available for analysis. Although the infant feeding practice information was collected at the same time as the stool sample collection and can demonstrate the impact of short-term exposures on the infant gut microbiota composition, we are unable to determine how these factors contribute to the temporal development of the infant gut microbiome. Also, we did not collect more detailed information, such as dose of vitamin D, maternal vitamin D status, timing of antibiotic administration. Future studies would benefit from a longitudinal stool sample collection during infancy and a more detailed infant feeding practices questionnaire that not only collects proximal but also long-term data about infant nutritional exposures.

APPENDIX

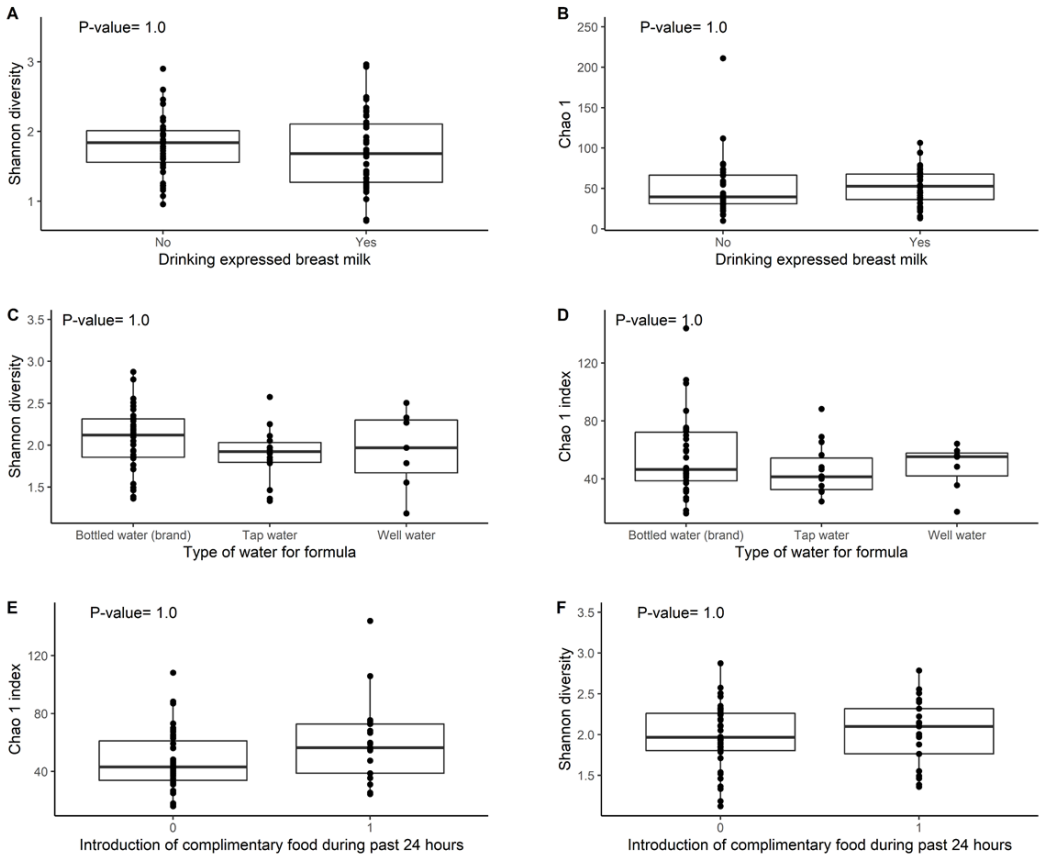

Figure S3.1. Infant alpha diversity by infant different feeding practices. FDR adjusted p-value for alpha diversity was displayed in upper-left. (A) The Shannon diversity was used for alpha diversity. Only exclusively breastfed infants were included in this subgroup analysis (N=92). Group differences were tested by Wilcoxon rank test. (B) The Chao 1 index was used for alpha diversity. Only exclusively breastfed infants were included in this subgroup analysis (N=92). (C) The Shannon diversity was used for alpha diversity. Only no breastfed infants were included in this subgroup (N=60). Group differences were tested by Kruskal-Wallis test. We then performed post hoc test for multiple comparisons and no significant associations was found. (D) The Chao 1 index was used for alpha diversity. Only no breastfed infants were included in this subgroup (N=60). Group differences were tested by Kruskal-Wallis test. We then performed post hoc test for multiple comparisons and no significant associations was found (N=92). (E) The Shannon diversity was used for alpha diversity. Only no breastfed infants were included in this subgroup (N=60). Group differences were tested by Kruskal-Wallis test. We then performed post hoc test for multiple comparisons and no significant associations was found. (F) The Chao 1 index was used for alpha diversity. Only no breastfed infants were included in this subgroup (N=60). Group differences were tested by Kruskal-Wallis test. We then performed post hoc test for multiple comparisons and no significant associations was found (N=60).

Table S3.1. Association between Shannon/Chao1 index and breastfeeding status in all infants[1]

	Shannon index		Chao 1 index	
	Beta (95% CI)	p-value	Beta (95% CI)	p-value
Breastfeeding status				
Exclusive breastfeeding	ref	-	ref	-
Partial breastfeeding	0.04 (-0.12, 0.20)	0.65	-0.32(-12.6, 6.3)	0.52
No breastfeeding	0.18 (0.01, 0.34)	0.03	0.02 (-9.6, 9.68)	0.98

[1]A multivariate linear regression regression analysis was performed to assess the association. Covariates in the models include maternal age, maternal education level, pre-pregnancy BMI, delivery mode, and infant age.

Table S3.2. Association between Shannon/Chao1 index and infant vitamin D supplement intake[1]

	Shannon index		Chao 1 index	
	Beta (95% CI)	p-value	Beta (95% CI)	p-value
Vitamin D supplement for infant				
No	ref	-	ref	-
Yes	-0.27 (-0.48, -0.08)	0.006	-10.8 (-22.08, 0.54)	0.06

[1]A multivariate linear regression regression analysis was performed to assess the association. Covariates in the models include maternal age, maternal education level, pre-pregnancy BMI, delivery mode, and infant age.

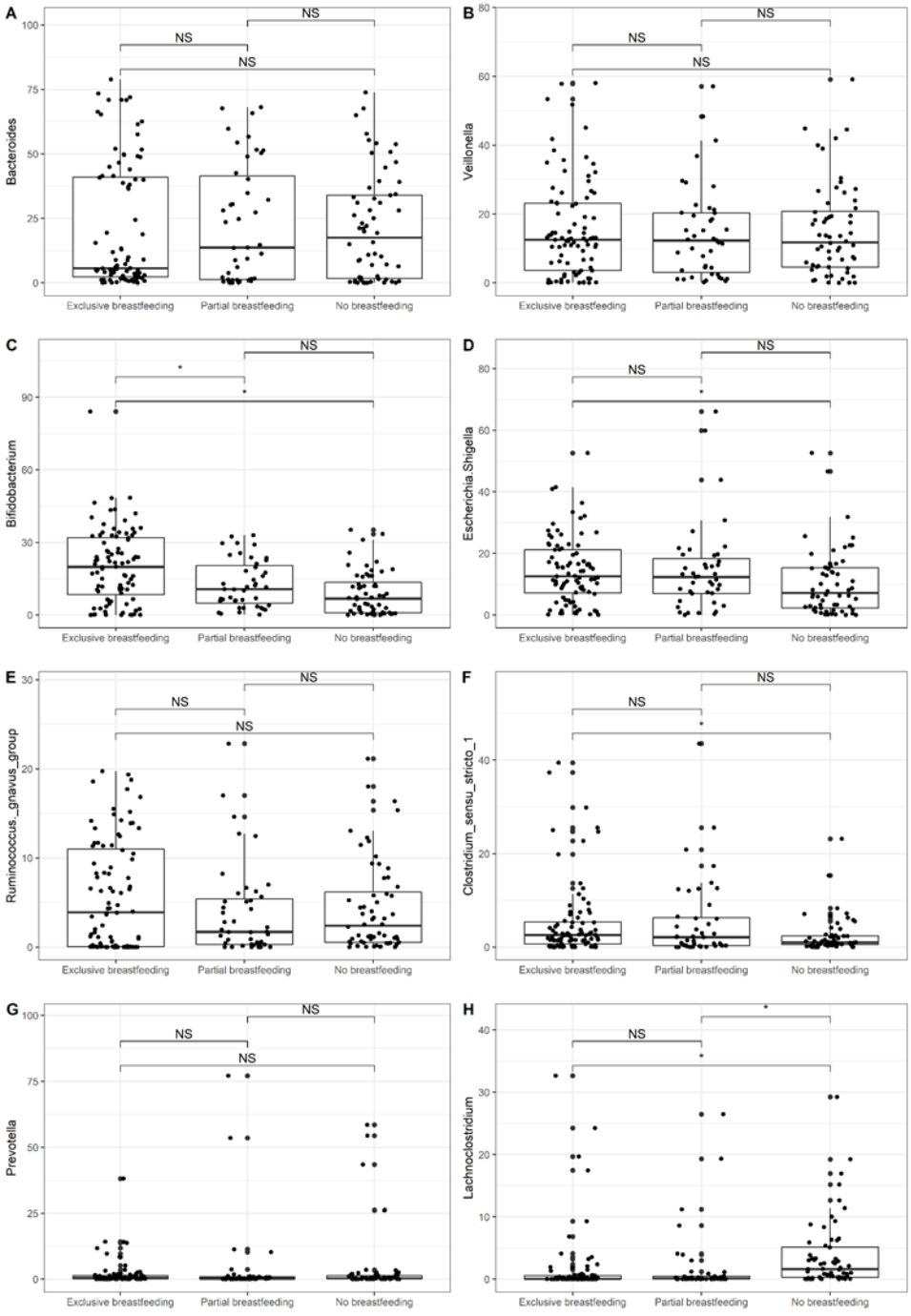

Figure S3.2. Association between breastfeeding status and relative abundance of 8 dominant genera. Group differences were tested by Kruskal-Wallis test. Wilcoxon rank test was used for post hoc test. P-value was adjusted by Bonferroni correction.
* indicates the adjusted p-value <0.05
NS indicates the adjusted p-value ≥ 0.05

Table S3.3. Results of Permutational Multivariate Analysis of Variance (PERMANOVA) on weighted UniFrac distances

Variable	F value	R^2	p-value
Breastfeeding during past week	4.8	4.70%	0.001*
Gestational age	4.1	2.10%	0.007*
Infant sex	1.7	0.80%	0.14
Delivery mode (virginal vs C-section)	6.0	3.00%	0.001*
Baby weight at delivery	0.4	0.20%	0.85
Infant probiotic supplement during past 24 hours	0.7	0.40%	0.57
Infant had any antibiotics since birth	0.9	0.90%	0.50
Maternal educational level	2.3	1.10%	0.06
Maternal pre-pregnancy BMI (continuous)	0.3	0.10%	0.95

* indicates the p-value <0.05

Table S3.4. Results of Permutational Multivariate Analysis of Variance (PERMANOVA) for exclusively breastfed infants on Bray-Curtis distances

Variable	F value	R^2	p-value
Gestational age	1.1	1.20%	0.31
Infant sex	0.9	1.00%	0.46
Delivery mode (virginal vs C-section)	3.0	3.50%	0.01*
Baby weight at delivery	0.3	0.40%	0.92
Infant probiotic supplement during past 24 hours	1.0	1.10%	0.41
Infant had any antibiotics since birth	0.8	0.90%	0.54
Maternal educational level	0.7	0.80%	0.67
Maternal pre-pregnancy BMI (continuous)	0.3	0.30%	0.95
Infant vitamin D supplement in the past 24 hours	2.9	3.40%	0.02*
Feeding with expressed breast milk	0.3	0.30%	0.95

* indicates the p-value <0.05

Table S3.5. Results of Permutational Multivariate Analysis of Variance (PERMANOVA)[1] for not breastfed infants on Bray-Curtis distances

Variable	F value	R^2	p-value
Gestational age	1.1	2.00%	0.31
Infant sex	1.2	2.10%	0.29
Delivery mode (virginal vs C-section)	1.3	2.30%	0.21
Baby weight at delivery	0.4	0.70%	0.94
Infant probiotic supplement during past 24 hours	0.7	1.20%	0.68
Infant had any antibiotics since birth	0.4	0.70%	0.95
Maternal educational level	2.4	4.10%	0.02*
Maternal pre-pregnancy BMI (continuous)	0.6	1.10%	0.75
Water type for formula	1.1	7.40%	0.39
Introduction of complementary food	0.8	1.40%	0.66

* indicates the p-value <0.05

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CHAPTER 4. ASSOCIATION OF THE INFANT GUT MICROBIOME WITH TEMPERAMENT

4.1 Abstract

Recent studies in both animals and humans have shown that gut microbiota is linked to brain development and control of behavior, but the association between gut microbiota and behavior in healthy infants is largely unknown. We undertook this prospective study to determine associations between gut microbiota at 3 months during infancy and infant temperament in a populational-based birth cohort. We analyzed data from 157 infants from the Michigan Archive for Research on Child Health Cohort Study. Infant temperament outcomes were reported by mothers using the Rothbart Infant Behavior Questionnaire-Revised Very Short Form at a mean age of 9.1 months. Microbiota profiling with 16S rRNA gene sequencing was conducted on fecal samples obtained at approximately 3 months of age. We identified three clusters of infants based on the relative abundance of gut microbiota: cluster A was characterized by a higher abundance of *Bacteroides*; cluster C was characterized by a higher abundance of *Bifidobacterium, Veillonella,* and *Escherichia-Shigella*; cluster B is intermediate between the other two clusters. Fully adjusted multivariate linear regression analysis showed a negative association between cluster C and negative emotionality score (coefficient = -0.58, p-value= 0.02) compared to cluster A, prominently among infants who were not given vitamin D supplement. However, no associations were evident between gut microbiota clusters and temperament scales after FDR correction. MaAslin analysis identified that individual microbial taxa were associated with three scales of temperament. This study found an association between infant gut microbiota composition and temperament by connecting temperament to microbiota clusters and individual taxa.

4.2 Introduction

The human gastrointestinal tract is the habitat of trillions of microorganisms that are closely associated with many aspects of human health, including physiological functions, metabolism, and immune functions[1–3]. A critical time for the microorganism, typically referred to as the microbiota, is during the first year of life since the gastrointestinal tract moves from a sterile to a bacteria rich environment[4]. The gut microbiota does not only aid human health physically but also plays a critical role in neurodevelopment through the interaction between microbiota and the brain, also known as the gut-brain axis[5,6]. The gut-brain axis hypothesizes that the brain and the microbiota communicate with each other through various chemical processes, which impact psychological and mental health[7].

The bidirectional relationship between the brain and the microbiota, or gut-brain axis, has been established using animal models. Previous studies have demonstrated that altered gut microbiota can lead to higher stress responsiveness[8], anxiety-like behaviors, abnormal social behaviors[9], and autism spectrum disorder behaviors[10]. Several studies have shown that germ-free (GF) mice who have no commensal microbiota and an undeveloped immune system[11,12] displayed increased motor activity and reduced anxiety compared with specific pathogen-free (SPF) mice with normal gut microbiota[13]. The abnormalities could be partially reversed when the gastrointestinal tracts of the germ-free mice were reconstituted with stool from normally raised mice[14]. Besides the behavioral differences, the brains of GF mice displayed various molecular differences, including brain-region-specific changes in levels of brain-derived neurotrophic factor, oxytocin, and vasopressin expression[15].

There is mounting evidence that gut microbiota plays a critical role in brain development and the control of behavior in humans[16,17]. Cross-sectional studies have discovered an association

between gut microbiome composition and neuropsychiatric outcomes, including autism spectrum disorder (ASD) and depression[18–20]. The disturbance of the gut microbiota in early life can lead to adverse mental health outcomes later in life[21]. Previous studies reported that children with ASD had lower abundances of *Coprococcus*, *Prevotella*, and unclassified *Veillonellaceae* compared to neurotypical children[22]. A recent study also reported a significant increase in the Firmicutes/Bacteroidetes ratio in children with ASD[23]. However, there are also some studies that observed no difference regarding severity ASD in the bacterial composition among children[24,25]. In terms of infant physical development, Sordillo et al. reported that infant gut microbiome composition at 3–6 months was associated with fine motor skills assessed by the Ages and Stages Questionnaire[26]. In a longitudinal study of 201 children, Loughman et al. found that the decreased abundance of the genus *Prevotella* in fecal samples collected at 12 months of age was associated with increased behavioral problems at age 2[27]. These studies suggest that the effects of the microbiome in early infancy play a more permanent role over the course of human life, whether it is in brain development or future behavioral outcomes.

The microbiome plays an essential role in behavior which determines temperament, a constitutionally based individual difference in emotion, motor behavior, attention, and self-regulation[28,29]. Longitudinal studies have reported that temperament characteristics in early life are associated with psychiatric problems, including anxiety, depression, ADHD, and autism in mid-childhood[30–32]. Despite the importance of temperament characteristics, few prospective studies have examined the associations between gut microbiome composition and temperament in infancy. In this study, we aimed to investigate the association between the fecal microbiota composition at 3 months of age and infant temperament at 9 months of age.

4.3 Materials and Methods

4.3.1 Study participants

The study population was drawn from the Michigan Archive for Research on Child Health (MARCH) cohort[33], an ongoing pregnancy and birth cohort set in Michigan's lower peninsula. The purpose of the MARCH study is to store biological specimens, and other health information that can be used to better understand the causes of problems in pregnancy and the health of children and to contribute to a nation-wide study of child health called the Environmental Influences on Child Health Outcomes (ECHO)[34]. Our analysis included mothers who provided informed consent for providing infant stool samples. During the MARCH 3 month phone interview, mothers confirmed their interest in participating in this sample collection. Fecal collection kits were sent by mail. 157 samples collected from singleton infants were included in the analysis.

4.3.2 Data collection

At approximately nine months of age, the Rothbart Infant Behavior Questionnaire-Revised Very Short Form (IBQ-RVSF) was administered to mothers by phone interview to assess the infant temperament[35]. The Infant Behavior Questionnaire is a widely used parent-report measure of infant temperament, first introduced by Rothbart in 1981[36,37]. IBQ-RVSF consists of 37 items measuring three dimensions of infant temperament, including positive affect/surgency (PAS, 13 items), negative emotionality (NEG, 12 items), and orienting/regulatory capacity (ORC, 12 items)[35]. Each item asked caregivers to report how often their babies engaged in a particular behavior during the last seven days. The items were rated on a scale ranging from 1 (never) to 7 (always). Higher scores of each scale indicate more of the measured temperament characteristic. PAS is characterized by positive loadings on approach, vocal reactivity, high intensity pleasure,

smiling and laughter, activity level, and perceptual sensitivity. NEG is analogous to the personality trait of neuroticism, and is characterized by positive loadings on sadness, distress to limitations, and fear, as well as negative loadings on falling reactivity. ORC is characterized by duration of orienting, low intensity pleasure, cuddliness, and soothability. Previous studies have demonstrated IBQR–VSF has adequate internal consistency, test-retest reliability, and interrater agreement between mothers and fathers[35]. Besides the IBQ-RVSF, multiple questionnaires were administered to mothers from the first prenatal visit through 9 months postpartum to collect a variety of socioeconomic and feeding practices factors. Detailed information on the neonate, including sex, birth weight, and gestational age, was abstracted from the birth certificate.

4.3.3 Fecal microbiota analysis

The fecal samples were aliquoted into sterile tubes and stored at -80°C, once received in the lab. DNA was extracted following a modified version of the Human Microbiome Project's protocol as described previously[38]. Barcoded primers were used to amplify the V4 region of the 16S rRNA gene region. The resulting 16S rRNA libraries were sequenced using 250 base pair Illumina MiSeq with V2 chemistry at the MSU genomics core. After trimming, clean sequences were analyzed using the QIIME2 (2021.2 version) pipeline[39]. QIIME2's DADA2 plugin was used to process the demultiplexed sequences and generate the amplicon sequence variants (ASV) table[40]. ASVs were assigned to taxonomy by the QIIME2 feature-classifier plugin, using the Silva 132 database at the similarity threshold of 99% (for 16S data)[41,42]. Samples were rarefied to 6,000 sequencing reads per sample, and taxa present in less than one sample were excluded, leaving 157 stool samples with 6,905 unique ASVs. ASVs were summarized at the genus taxonomic level.

4.3.4 Statistical analysis

All the analyses were performed using R software (version 4.0). The Shannon index, which represents microbial richness and evenness, and the Chao 1 index, which represents microbial richness, were calculated using the "vegan" package[43]. Associations between Shannon index or Chao1 index to temperament scales were assessed by multivariate linear regression models, adjusted by delivery mode (vaginal vs C-section), race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant sex, and infant age at IBQ-RVSF collection. Dirichlet multinomial mixture (DMM) clustering is an unsupervised Bayesian clustering method to identify clusters or enterotypes of microbial community data[44,45], as performed previously[46,47]. The best fitting DMM model was determined using the Laplace approximation. The difference of alpha diversity and relative abundance of taxa between DMM clusters were tested by Kruskal–Wallis test with Dunn test for post hoc. Multiple comparisons were adjusted for false discovery rate (FDR) correction using the Benjamini-Hochberg procedure. We performed Principal Coordinates Analysis (PCOA) and Permutational Multivariate Analysis of Variance (PERMANOVA) based on Bray–Curtis dissimilarity using the "vegan" package to compare the difference in microbial community structure between DMM clusters. We used multivariate linear regression models to determine the association between DMM clusters and infant temperament scales, adjusting by race, maternal education level, pre-pregnancy weight, delivery mode (vaginal vs C-section), infant sex, infant age at IBQ-RVSF collection, breastfeeding status, and infant vitamin D supplement.

Previous studies discovered that vitamin D and infant sex play roles as effect modifiers in the association between gut microbiota and neurodevelopment[48,49]. So, we conducted the sensitivity analyses to confirm the potential for infant vitamin D and sex to modify gut microbiome associations with temperament scales by stratification. we utilized MaAsLin, a

multivariate statistical framework that identifies associations between clinical metadata and individual microbiome abundance[50,51]. MaAslin provides both nominal p-values and FDR adjusted p-values (q-values) by Benjamini–Hochberg procedure. Associations were considered significant if the q-value was below the threshold of 0.2 in MaAslin results.

4.4 Results

4.4.1 Study Population Characteristics and temperament scales

157 participants were included in the final analysis (male= 52.2%, female= 47.8%). Descriptive statistics for maternal factors, infant factors by the three scales of infant temperament were displayed in Table 4.1. More than half of the mothers (58.6%) earned a college graduate degree, and 66.2% had a vaginal delivery. 57.3% of the mothers fed their children with exclusive breast milk at the time of fecal sample collection, and 92.4% of them had ever fed their children with breast milk. The mean (SD) age at temperament measurement was 9.1 (0.7) months, and the median was 9.0 (range: 8.0-13.0) months. Race is significantly associated with all three scales. Infants of white race were reported to have significantly lowest scores of all three scales, while infants of black race were reported to have significantly highest scores of NEG and ORC. Male has significantly higher PAS score than female (p-value <0.001). Higher maternal age is significantly associated with lower PAS score (coefficient = -0.03, p-value= 0.006) and lower NEG score (coefficient = -0.04, p-value= 0.009).

Table 4.1. Characteristics of mothers and infants by infant temperament scales

Scale		Positive affect/surgency		Negative emotionality		Orienting/regulatory capacity	
Variable	n (%) or mean (SD)	Mean (SD) or β (95% CI)	p-value	Mean (SD) or β (95% CI)	p-value	Mean (SD) or β (95% CI)	p-value
Categorical variable[1]							
Delivery mode, n (%)							
Vaginal delivery	104 (66.2%)	5.53 (0.65)	0.71	4.05 (0.96)	0.31	5.34 (0.68)	0.24
C-section	53 (33.8%)	5.58 (0.70)		4.22 (0.99)		5.48 (0.70)	
Maternal education level, n (%)							
Did not finish high school	4 (2.6%)	5.92 (0.98)	0.18	4.92 (1.05)	0.22	4.85 (1.10)	0.004*
High school graduate or GED	22 (14.0%)	5.75 (0.69)		4.30 (1.0)		5.81 (0.53)	
Some college	39 (24.8%)	5.59 (0.67)		4.14 (0.95)		5.46 (0.70)	

Table 4.1. (cont'd)

College graduate or more	92 (58.6%)	5.46 (0.64)		4.02 (0.96)		5.28 (0.67)	
Race, n (%)							
White	126 (80.3%)	5.46 (0.63)	<0.001*	4.02 (0.94)	0.03*	5.29 (0.69)	0.002*
Black	20 (12.7%)	5.82 (0.73)		4.53 (0.88)		5.85 (0.58)	
Other	11 (7.0%)	6.04 (0.70)		4.42 (1.25)		5.66 (0.56)	
Breastfeeding status at fecal sample collection, n (%)							
Exclusive breastfeeding	90 (57.3%)	5.51 (0.67)	0.37	4.17 (0.95)	0.68	5.29 (0.70)	0.1
Partial breastfeeding	41 (26.1%)	5.72 (0.63)		4.03 (0.96)		5.60 (0.75)	
Not breastfeeding	26 (16.6)	5.53 (0.68)		4.03 (1.04)		5.46 (0.62)	
Ever breastfeeding, n (%)							
Yes	145 (92.4%)	5.53 (0.66)	0.32	4.09 (0.97)	0.38	5.38 (0.71)	0.73
No	12 (7.6%)	5.73 (0.70)		4.34 (1.02)		5.46 (0.51)	

Table 4.1. (cont'd)

Baby sex, n (%)							
Male	82 (52.2%)	5.72 (0.64)	<0.001*	4.14 (0.96)	0.69	5.40 (0.73)	0.77
Female	57 (47.8%)	5.36 (0.65)		4.08 (0.98)		5.37 (0.65)	
Continuous variable[2]							
Maternal age (year), mean (SD)	31.5 (5.0)	-0.03 (-0.05, -0.008)	0.006*	-0.04 (-0.07, -0.01)	0.009*	-0.006 (-0.03, 0.02)	0.61
Pre-pregnancy BMI, mean (SD)	27.8 (7.2)	0.01 (-0.005, 0.02)	0.18	0.001 (-0.02, 0.02)	0.96	0.01 (-0.002, 0.03)	0.09
Gestational age at delivery (week), mean (SD)	38.8 (1.5)	0.03 (-0.04, 0.10)	0.38	0.02 (-0.08, 0.13)	0.67	-0.05 (-0.13, 0.02)	0.14
Infant age at IBQ measurement (month), mean (SD)	9 (0.7)	0.004 (-0.001, 0.008)	0.14	0.04 (-0.003, 0.01)	0.32	0.002 (-0.004, 0.007)	0.55

[1]Summary of categorical variables were displayed as n (%). The difference in temperament scores between categorical variables was analyzed by One-Way Analysis of Variance (ANOVA).
[2]Summary of continuous variables were displayed as mean (SD). The association between continuous variables and temperament scales was analyzed by linear regression.
*p-value < 0.05

4.4.2 Alpha diversity and temperament scores

Table 4.2 presents the association between alpha diversity of fecal samples and infant temperament scales. Higher Shannon index is associated with higher positive affect/surgency scores (coefficient=0.16, p-value= 0.12) and lower negative emotionality score (coefficient= -0.16, p-value= 0.27), but the associations were not statistically significant. We also did not observe any significant associations between Chao 1 index and temperament scales (Table 4.2).

Table 4.2. Association between alpha diversity and infant temperament[1]

	Shannon		Chao 1	
Scale	Beta	p-value	Beta	p-value
Positive affect/surgency	0.13	0.18	0.001	0.44
Negative emotionality	-0.20	0.20	-0.004	0.08
Orienting/regulatory capacity	0.003	0.98	-0.001	0.66

[1]Linear regression Models were adjusted for delivery mode, race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant sex, and infant age at IBQ-RVSF collection.

4.4.3 Cluster analysis

We employed Dirichlet multinomial mixture (DMM) modeling to assign microbiota composition into clusters. Using the minimum Laplace approximation, we identified three optimal clusters, which we also referred to as enterotypes (Figure 4.1A). Cluster A, B, and C accounted for 25%, 56%, and 19% of the total samples, respectively. A significantly lower Shannon index and Chao 1 index were observed in cluster B, compared to cluster A and C (Figure 4.1B-4.1C). In addition, the gut microbiota communities were well-separated by the three clusters in principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix (univariate PERMANOVA: R^2= 11.9%, p-value =0.001, Figure 4.1D). Thus, these results revealed broad community differences

across the three clusters. Figure 4.1E displayed the heatmap, which shows the relative abundance of the top ten genera within each cluster. Samples in each cluster were ordered by the relative abundance of *Bacteroides*. *Bacteroides, Bifidobacterium, Veillonella,* and *Escherichia-Shigella* were the top 4 genera that drove the clustering (Figure 4.2). Samples in cluster A exhibited a higher relative abundance of *Bacteroides* than the cluster C (adjusted p-value = 1.7×10^{-10}), but similar abundance of *Bacteroides* to cluster B (adjusted p-value= 0.33). Cluster C was characterized by the highest relative abundance of *Bifidobacterium, Veillonella,* and *Escherichia-Shigella,* compared to the cluster A and B.

To investigate the potential association between clusters and infant temperament, we applied linear regression models, adjusted by covariates including delivery mode (vaginal vs C-section), race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant vitamin D intake, infant sex, and infant age at IBQ-RVSF collection (Table 4.3). The results of univariate models demonstrated that cluster C is significantly associated with lower PAS score (coefficient = -0.43, p-value=0.009), and lower NEG score (coefficient = -0.54, p-value= 0.02), comparing to the cluster A. In multivariate linear regression models, cluster C is significantly associated with a lower NEG score (coefficient = -0.58, p-value=0.02), comparing to cluster A. However, after FDR correction, none of these clusters remained significantly associated with the temperament scales.

In the sensitivity analysis, among the infants who had not taken any vitamin D supplements, infants of cluster C showed a significantly lower NEG scores than cluster A (coefficient = -1.01, p-value = 0.01) before FDR correction (Table S4.1). While in the vitamin D group, cluster C was no longer significant with NEG score, and displayed less pronounced coefficient (coefficient = -0.43, p-value = 0.20), which suggested the associations were potentially attenuated by the

vitamin D intake. We did not find any significant association between gut microbiota clusters and temperament scales when stratifying by infant sex (Table S4.2).

4.4.4 Individual taxa analysis

We next sought to assess the association between individual taxa at the genus level and infant temperament scales using MaAslin (Table 4.4). Infants with higher positive affect/surgency scores had significantly higher relative abundance of a set of Firmicutes, including genera *Oscillospiraceae* UCG-003, *Oscillospiraceae* UCG-002, *Christensenellaceae* R-7 group. Infants with higher negative emotionality score had significantly lower relative abundance of *Lachnospiraceae* FCS020 group (MaAslin coefficient = -0.07, q-value= 0.012), and higher relative abundance of *Eggerthella* (MaAslin coefficient = 0.10, q-value= 0.13). Infants with higher orienting/regulatory capacity scores had a significantly lower relative abundance of *Clostridioides* (MaAslin coefficient = -0.14, q-value= 0.19).

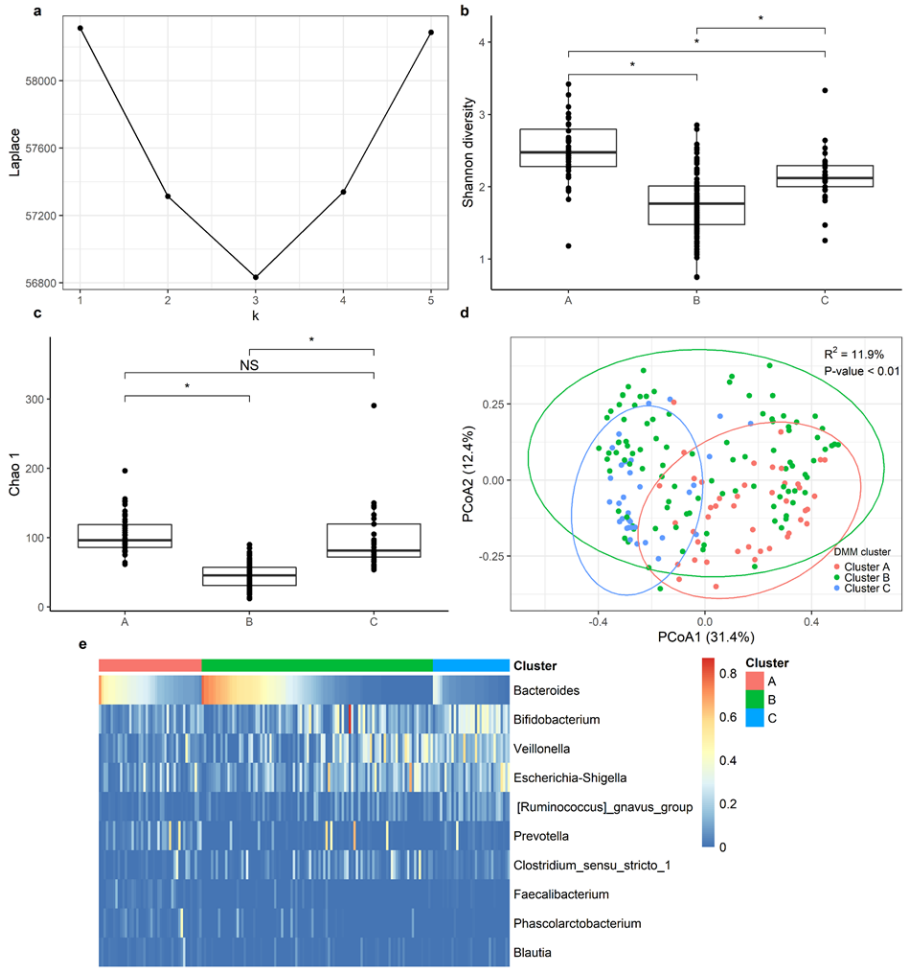

Figure 4.1. Dirichlet multinomial mixture clustering identified three optimal clusters from 157 fecal samples. (a) The number of clusters (k=3) was chosen by selecting the minimal Laplace approximation to the negative log model evidence. (b-c) Boxplot of the alpha diversity (Shannon and Chao 1) distributed between the 3 clusters. Group differences were tested by Wilcoxon signed-rank test, and p-values were adjusted for multiple testing using Bonferroni. Adjusted p-value <0.05 was labeled as *, and adjusted p-value ≥ 0.05 was labeled as NS. (d) Principal component analysis (PCoA) ordinations of variation based on the Bray–Curtis distance matrix. R^2 and p-value were calculated by the univariate PERMANOVA test. (e) Heatmap of relative abundance of top 10 genera by the three clusters. Within the clusters, samples were ordered by the relative abundance of the genus Bacteroides.

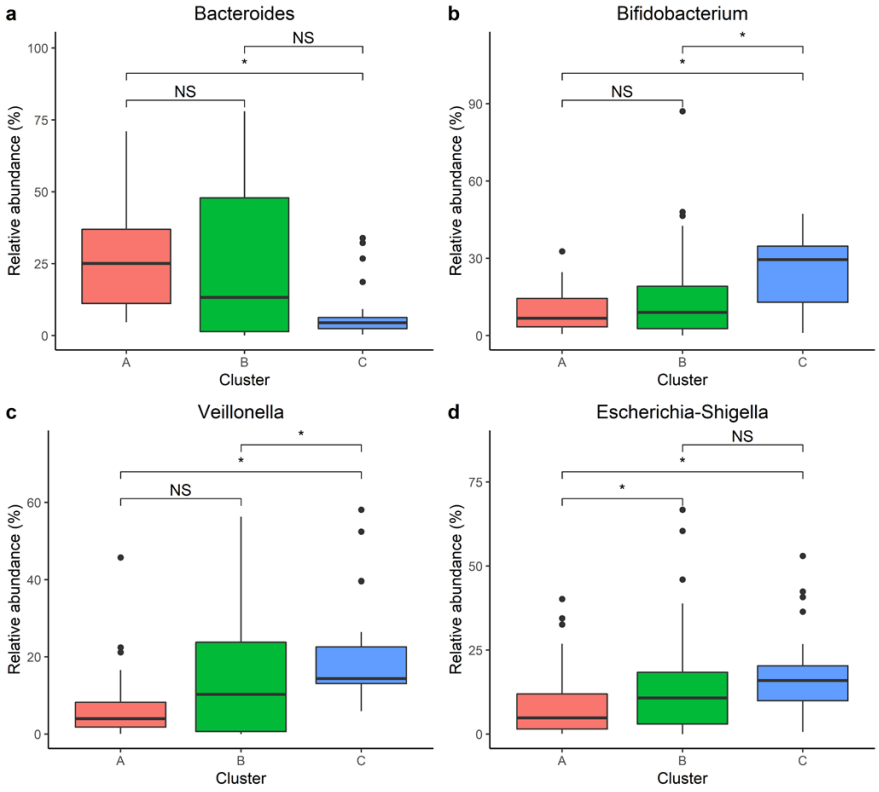

Figure 4.2. Relative abundance of the top 4 genera that contribute by clusters. Group differences were tested by Wilcoxon signed-rank test. P-values were adjusted for multiple testing using Bonferroni. Adjusted p-value <0.05 was labeled as *, and adjusted p-value ≥ 0.05 was labeled as NS.

Table 4.3. Association between gut microbiota clusters and infant temperament scales[1]

Scale	Cluster	Univariate model			Multivariate model		
		Beta	p-value	q-value	Beta	p-value	q-value[2]
Positive affect/surgency	Cluster A	ref	-	-	ref	-	-
	Cluster B	-0.20 (-0.46, 0.05)	0.12	0.18	-0.12 (-0.38, 0.13)	0.34	0.51
	Cluster C	-0.43 (-0.74, -0.11)	0.009*	0.05	-0.29 (-0.61, 0.02)	0.07	0.21
Negative emotionality	Cluster A	Ref	-	-	ref	-	-
	Cluster B	-0.04 (-0.41, 0.33)	0.84	0.84	-0.04 (-0.43, 0.34)	0.82	0.82
	Cluster C	-0.54 (-1.00, -0.08)	0.02*	0.06	-0.58 (-1.05, -0.10)	0.02*	0.12
Orienting/regulatory capacity	Cluster A	ref	-		ref	-	-
	Cluster B	-0.09 (-0.36, 0.18)	0.53	0.64	-0.06 (-0.33, 0.22)	0.69	0.82
	Cluster C	-0.30 (-0.63, 0.04)	0.08	0.16	-0.21 (-0.56, 0.13)	0.22	0.44

[1]Models were adjusted for delivery mode, race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant vitamin D intake, infant sex, and infant age at IBQ-RVSF collection.
[2]P-values were adjusted by false discovery rate (FDR) correction for multiple comparisons using the Benjamini-Hochberg procedure.
*p-value < 0.05

Table 4.4. MaAsLin Analysis Results: Associations of gut microbiome taxa at genus level and infant temperament scales adjusted by covariates[1]

Scale	Phylum	Family	Genus	Coefficient	N/ N not 0	p-value	q-value[2]
Negative emotionality	Firmicutes	Lachnospiraceae	*Lachnospiraceae*_FCS020_group	-0.07	157/ 116	0.00003	0.013
	Actinobacteriota	Eggerthellaceae	*Eggerthella*	0.10	157/ 44	0.002	0.131
Positive affect/surgency	Firmicutes	Christensenellaceae	*Christensenellaceae* R-7 group	0.16	157/ 57	0.001	0.112
	Firmicutes	Oscillospiraceae	UCG-003	0.12	157/ 44	0.001	0.113
	Firmicutes	Oscillospiraceae	UCG-002	0.14	157/ 73	0.003	0.15
Orienting/regulatory capacity	Firmicutes	Peptostreptococcaceae	*Clostridioides*	-0.14	157/ 79	0.009	0.20

[1]Model was adjusted for delivery mode, race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant vitamin D intake, infant sex, and infant age at IBQ-RVSF collection.
[2]Q-value is the FDR (Benjamini-Hochberg) adjusted p-value. Q-value < 0.2 for multiple comparisons was considered statistically significant and included in the table.

4.5 Discussion

Accumulating evidence from animal and human studies report that the gut microbiota plays a role in neurodevelopment during the early critical time window. In the current study, we focus on the three subscales of the infant temperament: positive affect/surgency, negative emotionality,

and orienting/regulatory capacity. Our results show an association between infant gut microbiota composition and temperament by connecting temperament to both DMM clusters and individual taxa.

4.5.1 Alpha diversity and temperament scales

No significant associations were found between alpha diversity and temperament scales. This finding is consistent with some results of studies from longitudinal and cross-section studies. Fox et al. investigated the relationship between gut microbiota at each age group (1–3 weeks, 2, 6, and 12 months) and IBQ scores at 12-months of age[52]. No temperament scales at age 12 months demonstrated a significant association with the alpha diversity measures at each age. Similarly, Kelsey et al. demonstrated that neither alpha diversity nor richness was associated with any of the temperament scales in a cross-sectional study[53].

4.5.2 Cluster analysis

We identified three clusters based on the DMM method. Cluster A was characterized by a higher abundance of *Bacteroides*; cluster C is characterized by a higher abundance of *Bifidobacterium*, *Veillonella*, and *Escherichia-Shigella*; while cluster B is intermediate between the other two clusters. Our result shows that cluster C had a significant inverse association with negative emotionality. Negative emotionality, which is defined by the disposition to experience negative emotions such as anger and fear[54], has often been linked with internalizing and externalizing problems[55,56]. Previous work by Aatsinki et al. also showed the same trend toward the relationship between Bifidobacterium-dominated/ Bacteroides-dominated cluster and negative emotionality, though it was not statistically significant[57]. In addition, two studies of early infants discovered the association between Bacteroides-dominant community and poor fine

motor skills, which also supports the adverse effect of Bacteroides-dominated microbiome composition on infant neurodevelopment[47,58].

In the sensitivity analysis, we discovered the potential role of infant vitamin D intake in modifying the association between clusters and negative emotionality. The association was only identified among the infants who were not recipients of vitamin D supplements, suggesting infant vitamin D intake potentially protects against the adverse effect of the gut microbiome–associated increments on infant negative emotionality. Similarly, a prior study reported that in participants who received prenatal vitamin D treatment, the Veillonella-dominated gut microbiota community was associated with improved communication scores in infants, whereas no association between the Veillonella-dominated community and communication scores was observed in the control group[58]. A possible explanation is that, vitamin D and vitamin D receptor (VDR) levels modify gut microbiota in neurodevelopment through the gut–microbiota–brain axis, such as cytokines, neurotransmitters, and SCFAs[59].

4.5.3 Individual taxa

Our results showed that several taxa were significantly associated with infant temperament outcomes, suggesting a mechanism of influence on infant temperament involving specific taxa of the gut microbiome. Of note, our results agree with the previous literature, which suggests that the genus *Clostridioides* (*Clostridium*) in the infant's gut is associated with adverse neurodevelopment outcomes[57,60]. *Clostridioides* is demonstrated to be an important predictor of infant temperament, as a similar study also reports a significant negative association between *Clostridioides* at age 2.5 months and regulation scale at age 6 months[57]. *Clostridioides difficile* (*C difficile*), which can cause severe diarrhea and colitis, is the most common *Clostridioides* species in the infant. Animal studies showed that *C. difficile* can produce Propionic Acid, a short-

chain fatty acid that can introduce ASD-related symptoms[61,62]. Future research should further examine the specific strain of *Clostridioides* in the infant gut to confirm the association between C difficile and infant temperament. Our results did not identify any significant associations between *Bifidobacterium* and positive affect/surgency, which is in contrast to other studies. Both Fox et al. and Aatsinki et al. reported that *Bifidobacterium* abundance at early infancy was positively associated with surgency/extraversion[52,57]. Bifidobacterium is typically considered as a beneficial bacteria for infant neurodevelopment, demonstrating benefits for gut epithelium integrity and function as well as gastrointestinal motility[63]. Larger sample size may need for our study to replicate the results from previous studies.

4.5.4 Strength and limitation

Our current study may have a number of strengths. We demonstrated prospective associations between infant gut microbiome and temperament in a population-based cohort study. In addition to adjustment for prenatal and infant variables, our models also accounted for the mode of delivery, gestational age, and nutrition. The associations between microbiome clusters and negative emotionality are robust to adjustment for these variables. Our analysis not only demonstrated the effect of gut microbiota composition on infant temperament but also highlighted the importance of individual taxa. Of note, our study is not free of limitations. First, infant gut microbiota is rapidly maturated over the first year of life. However, only one fecal sample was included in our analysis and cannot fully represent the temporal development of the infant gut microbiome, which may be critical to neurodevelopment in early life. Second, 16S rRNA sequencing can only provide very limited strain-level information. Previous studies demonstrate the biological importance and different metabolic capabilities of specific bacterial

strains on human health. Thus, Shotgun metagenomic sequencing is needed for our future studies.

APPENDIX

Table S4.1. Association between gut microbiota clusters and infant temperament scales stratifying by infant vitamin D intake[1]

Scale	Cluster	No Vitamin D			Vitamin D		
		Beta	p-value	q-value[2]	Beta	p-value	q-value
Positive affect/surgency	Cluster A	ref	-		ref	-	
	Cluster B	-0.34 (-0.86, 0.18)	0.20	0.46	-0.05 (-0.35, 0.25)	0.76	0.91
	Cluster C	-0.33 (-0.87, 0.20)	0.23	0.46	-0.35 (-0.77, 0.08)	0.11	0.46
Negative emotionality	Cluster A	ref	-		ref	-	
	Cluster B	-0.50 (-1.21, 0.22)	0.18	0.46	0.03 (-0.43, 0.49)	0.89	0.91
	Cluster C	-1.01 (-1.75, -0.26)	0.01*	0.12	-0.43 (-1.08, 0.23)	0.20	0.46
Orienting/regulatory capacity	Cluster A	ref	-		ref	-	
	Cluster B	-0.03 (-0.54, 0.48)	0.91	0.91	-0.15 (-0.44, 0.39)	0.38	0.57
	Cluster C	-0.13 (-0.66, 0.40)	0.63	0.84	-0.23 (-0.90, 0.25)	0.34	0.57

[1]Models were adjusted for delivery mode, race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant age at IBQ-RVSF collection and infant sex.

[2]P-values were adjusted by false discovery rate (FDR) correction for multiple comparisons using the Benjamini-Hochberg procedure.

*p-value < 0.05

Table S4.2. Association between gut microbiota clusters and infant temperament scales stratifying by infant sex[1]

Scale	Cluster	Male			Female		
		Beta	p-value	q-value[2]	Beta	p-value	q-value
Positive affect/surgency	Cluster A	ref	-		ref	-	
	Cluster B	-0.08 (-0.42, 0.26)	0.63	0.83	-0.24 (-0.67, 0.19)	0.27	0.54
	Cluster C	-0.30 (-0.74, 0.14)	0.18	0.48	-0.41 (-0.91, 0.10)	0.12	0.48
Negative emotionality	Cluster A	ref	-		ref	-	
	Cluster B	0.04 ()	0.86	0.86	-0.13 (-0.74, 0.49)	0.69	0.83
	Cluster C	-0.59 ()	0.08	0.48	-0.51 (-1.23, 0.22)	0.18	0.48
Orienting/regulatory capacity	Cluster A	ref	-		ref	-	
	Cluster B	0.06 (-0.32, 0.44)	0.76	0.83	-0.20 (-0.64, 0.24)	0.38	0.60
	Cluster C	-0.33 (-0.82, 0.16)	0.20	0.48	-0.22 (-0.74, 0.30)	0.40	0.60

[1]Models were adjusted for delivery mode, race, maternal education, maternal pre-pregnancy BMI, breastfeeding status, infant age at IBQ-RVSF collection, infant vitamin D.

[2]P-values were adjusted by false discovery rate (FDR) correction for multiple comparisons using the Benjamini-Hochberg procedure.

*p-value < 0.05

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CHAPTER 5. ASSOCIATION BETWEEN INFANT GUT MICROBIOME AND SLEEP PROBLEMS DURING CHILDHOOD

5.1 Abstract

The difficulty of initiating and maintaining sleep are the two most predominant complaints of sleep disorders in childhood. To the best of our knowledge, no study has yet investigated the association between gut microbiota collected at early infancy and these two disorders in early childhood. We undertook this prospective study to determine the associations between gut microbiota collected at early infancy (3-9 months) and difficulty initiating and maintaining sleep at the age of 2 years in a populational-based birth cohort. We analyzed data from 195 infants from the Michigan Archive for Research on Child Health Cohort Study. Sleep disorders were abstracted from the items in the PROMIS Sleep Disturbance scale, which was reported by mothers. Feeding practices and clinical information were collected by questionnaire and abstraction of birth certificates. Microbiota profiling with 16S rRNA gene sequencing at the V4 region was conducted on fecal samples. Gut microbiota of children who had difficulty of maintaining sleep displayed significantly higher Shannon index (OR: 2.41, 95% CI= 1.23-4.93, p-adjust < 0.04) and Chao 1 index (OR: 1.01, 95% CI= 1.0-1.03, p-adjust < 0.008) after adjustment for covariates. We also demonstrated that gut microbiota composition was significantly associated with difficulty initiating (p-value= 0.043) and maintaining sleep (p-value= 0.004) based on the unweighted UniFrac distance metric in 2-year-old children. In the DESeq2 analysis for individual taxa, we identified several taxa associated with each of two sleep disorders at the genus level. This study demonstrated a clear association between infant gut microbiota and sleep disorders in early childhood.

5.2 Introduction

5.2.1 Sleep in childhood

Sleep quality and quantity are increasingly being considered critical factors for child health and development. Epidemiology studies indicate that up to 50% of children experience a sleep disorder between 0 to 6 years old[1–3]. The frequency of childhood sleep disorders from 2

general pediatric clinics indicated that 41% of parents reported insomnia in their children[4]. Difficulty initiating and maintaining sleep are the two most frequent sleep problems in childhood and often co-exist[5]. Multiple biological, psychosocial, and environmental factors are linked to sleep disorders in children[6]. Obesity is one of the critical factors associated with sleep disorders, and the relationship is bidirectional[7]. Cross-sectional studies from multiple countries suggested that increased levels of obesity are associated with decreased sleep duration[8–11]. Previous studies also have reported a higher prevalence and severity of obstructive sleep apnea (OSA) in children with obesity[12].

Environmental exposure also plays a vital role in sleep disorders among children. For example, elevated blood lead levels in early childhood are associated with increased risk for sleep disorders and excessive daytime sleepiness in later childhood[13]. Sleep disorders are associated with an increased risk of poor school performance, anxiety, depression, aggressive behaviors, and attention problems that continue in adulthood [14–17]. Sleep disorders interrupt the duration and depth of sleep, which are essential for child development. It is critical to identify the underlying factors involved in sleep disorders.

5.2.2 The gut microbiome and sleep

Disruption of the gut microbiota had been linked to psychiatric disorders and behavior problems in children. Growing evidence points toward the gut-microbiota-brain axis, which refers to the network involving multiple biological systems that allow bidirectional communication between gut bacteria and the brain[18]. The communication pathways in these biological networks include both direct and indirect signaling via chemical transmitters, neuronal pathways, and the immune system[18]. Sleep disorders, which are closely related to psychiatric disorders, have been linked to gut microbiota in recent years. Animal studies showed that during sleep

fragmentation, the relative abundance of Actinobacteria, *Lactobacillaceae,* and *Bifidobacteriaceae* decreased, while the relative abundance of Ruminococcaceae increased in mice[19,20]. A cohort study showed that children with Obstructive Sleep Apnoea syndrome were associated with a lower microbiota diversity in respect to healthy subjects[21]. A study among old adults also suggested a possible relationship between the composition of the gut microbiome and sleep quality[22].

Although difficulty initiating and maintaining sleep has a high prevalence among children, the mechanisms underlying the development of these two sleep problems has not yet been understood[23]. To our knowledge, no study has investigated their association with gut microbiota in children. Therefore, the present study aimed to investigate the association between the fecal microbiota collected in early infancy and difficulty initiating and maintaining sleep at the age of two years.

5.3 Materials and Methods

5.3.1 Study participants

The study population was drawn from the Michigan Archive for Research on Child Health (MARCH) cohort, an ongoing population-based pregnancy and birth cohort set in Michigan's lower peninsula. During the MARCH 3 month phone interview, mothers confirmed their interest in participating in this sample collection. Our analysis used the subset of the cohort whose mothers provided informed consent for providing infant stool samples. Fecal collection kits were sent by mail. The infants in this analysis were 3-9 months of age between 2018 and 2021 at time of fecal sample collection. 195 fecal samples collected from singleton infants were included in the analysis.

5.3.2 Data collection

Several questionnaires were administered to mothers from the first prenatal visit through 2 years postpartum. The questionnaire during the prenatal visit included demographic information about the mothers, their breastfeeding plans, health-related practices, and their estimated due date. Detailed information, including the infant's sex, birth weight, pregnancy complications, mode of delivery (vaginal vs C-section), pre-pregnancy BMI, and gestational age, were abstracted from the birth certificate.

To assess sleep problems of the children, we administered the PROMIS Sleep Disturbance scale (PSDS, four items)[24] to mothers when their children were age 2. PSDS has demonstrated high internal consistency and strong construct validity among children in previous studies[24,25]. For our outcomes, we used two items from the PSDS to represent difficulty initiating sleep (my child had difficulty falling asleep) and difficulty maintaining sleep (my child could not sleep through the night). The items followed a 5-point format (1= never, 2= almost never, 3= sometimes, 4= almost always, 5= always). We considered the children to be free of difficulty initiating or maintaining sleep if the scores reported by mothers were less or equal than 2.

5.3.3 Fecal microbiota analysis

Once received in the lab, the fecal samples were aliquoted into sterile tubes and stored at -80°C. DNA was extracted following a modified version of the Human Microbiome Project’s protocol described previously[26]. Barcoded primers were used to amplify the V4 region of the 16S rRNA gene following the mothur wet lab documentation. PCR amplification also followed the wet lab protocol outlined in the mothur documentation. The resulting 16S rRNA libraries were sequenced using 250 base pair Illumina MiSeq with V2 chemistry at the Michigan State University genomics core. After trimming, clean sequences were analyzed using the QIIME2 (2021.2 version) pipeline[27]. Demultiplexed sequences were further quality filtered and

clustered using QIIME2's DADA2 plugin to generate the ASV table[28]. Unique amplicon sequence variants (ASVs) were assigned a taxonomy by the QIIME2 feature-classifier plugin, using the Silva 132 database at the similarity thresh-old of 99% (for 16S data)[29,30]. Samples were rarefied to 6,000 sequencing reads per sample, leaving 191 stool samples with 6,905 unique ASVs, and findings were summarized at the genus taxonomic level.

5.3.4 Statistical analysis

We used multivariate logistic regression to assess the association between characteristics of the study population and sleeping disorders at age of two years, with adjustment for breastfeeding status, delivery mode, birth weight, gestational age, age at fecal sample collection. Characteristic variables included maternal education level, pre-pregnancy BMI, race, sex, and maternal age. Gut microbiota was analyzed in terms of alpha diversity (Chao1 and Shannon) and beta diversity (Unweighted UniFrac and Weighted UniFrac) using the "vegan" package in R[31]. The difference in alpha diversity and relative abundance of taxa between sleep outcomes were tested by the Wilcoxon rank test with false discovery rate (FDR) correction for multiple comparisons. We also performed multivariate logistic regression models to estimate the association between alpha diversity and sleep disorders, with adjustment for breastfeeding status, delivery mode, birth weight, gestational age, age at fecal sample collection, maternal education level, pre-pregnancy BMI, race, baby sex, and maternal age. We performed Principal Coordinates Analysis (PCoA) and Permutational Multivariate Analysis of Variance (PERMANOVA) based on Unweighted UniFrac and Weighted UniFrac distance metric to compare the difference in microbial community structure between sleep disorders[32,33]. PERMANOVA is a non-parametric multivariate statistical test, with p-values obtained using appropriate distribution-free permutation techniques. Differential abundance of microbial genera was determined using

generalized linear models with a negative binomial family and a log link function implemented in DESeq2[34], with adjustment by delivery mode, maternal education level, maternal age, gestational age, pre-pregnancy BMI, race, breastfeeding status, age at fecal sample collection, child sex, and birth weight. DESeq2 assumes that counts can be modeled as a negative binomial distribution with a mean parameter, allowing for size factors and a dispersion parameter[34].

5.4 Results

In total, 95 boys and 99 girls with data on sleep disturbance at age two years were included in this analysis. The mean age of the children when the PROMIS Sleeping Disturbance questionnaire was implemented to mothers was 24.2 (SD= 1.2) months. The mean age of the children at fecal sample collection was 4.0 (SD= 1.1) months. Mean birth weight was 3,352 (SD= 583) grams, and mean gestational age was 38.5 weeks (SD= 1.9). Mean (SD) maternal age was 30.9 (5.4) years and mean pre-pregnancy BMI was 27.9 (SD= 7.6). 100 children were exclusively breastfed, 33 were partially breastfed, and 61 were fed with exclusive formula at the time of fecal sample collection. At one year, 169 children were ever breastfed or fed with pumped breast milk and 25 were never fed with breast milk. Descriptive summary of study participants by difficulty initiating sleep and maintaining sleep are shown in Table 5.1.

Over the 194 children, 61 (31%) were reported to have difficulty initiating sleep, 63 (32.5%) were reported to have difficulty maintaining sleep, 32 (16.4%) were reported to have both disorders, and 92 (47.2%) were reported to have at least one disorder. Univariate analysis showed that a lower proportion of mothers whose children had difficulty initiating sleep were of normal BMI (< 25.0) prior to pregnancy compared to those whose children did not (36.1% vs 47.8%, p-value= 0.004). Children with difficulty maintaining sleep were more likely to be never fed with breast milk, compared to those who were ever fed with breast milk (22.2% vs 8.5%, p-

value= 0.01). In a multivariate model, children whose mothers were overweight were more likely to have difficulty initiating sleep (OR = 3.7, CI:1.6-8.7, p-adjust = 0.004) and more likely to have difficulty maintaining sleep (OR = 2.7, CI:1.2-6.4, p-adjust = 0.04) at age 2, compared to those whose mothers with normal BMI (Table S5.1-S5.2). Increased birth weight tended to be associated with a lower risk of having difficulty initiating sleep in infants (OR = 0.4, CI:0.2-0.9, p-adjust = 0.08, Table S5.2).

Table 5.1. Characteristics of mothers and infants by difficulty initiating sleep and maintaining sleep

	Difficulty initiating sleep				P-value [1]	Difficulty maintaining sleep				P-value
	Yes (61)		No (134)			Yes (63)		No (131)		
	N/mean	%/SD	N/mean	%/SD		N/mean	%/SD	N/mean	%/SD	
Delivery mode, n (%)										
Vaginal delivery	37	61%	98	74%		39	62.90 %	95	72.50 %	
C-section	24	39%	35	26%	0.1	23	37.10 %	36	27.50 %	0.24
Maternal education level, n (%)										

Table 5.1. (cont'd)

Did not finish high school	5	8.50%	8	6%		7	11.70%	6	4.60%	
High school graduate or GED	6	10.20%	18	13.50%		9	15.00%	15	11.50%	
Some college	13	22.00%	31	23.30%		15	25.00%	29	22.10%	
College graduate or more	35	59.30%	76	57.20%	0.85	29	48.30%	81	61.80%	0.17
Maternal pre-pregnancy BMI										
Normal (<25)	22	36.10%	64	47.80%		21	33.30%	64		
Overweight (25-30)	24	39.30%	23	17.20%		19	30.20%	28		
Obesity (≥ 30)	15	24.60%	47	35.00%	**0.004**	23	36.50%	39		0.12
Race, n (%)										
White	44	74.60%	105	78.90%		42	70.00%	106	80.90%	
Black	11	18.60%	20	15.00%		13	21.70%	18	13.70%	
Other	4	6.80%	8	6.10%	0.8	5	8.30%	7	5.30%	0.21

Table 5.1. (cont'd)

Breastfeeding status at fecal sample collection, n (%)										
Exclusive breastfeeding	31	51.70%	69	51.50%		27	43.50%	72	55.00%	
Partial breastfeeding	11	18.30%	22	16.40%		10	16.10%	23	17.60%	
Not breastfeeding	18	30%	43	32.10%	0.92	25	40.40%	36	27.40%	0.19
Ever exposed to breast milk, n (%)										
Yes	53	86.90%	116	87.20%		49	77.80%	119	91.50%	
No	8	13.10%	17	12.80%	0.99	14	22.20%	11	8.50%	**0.01**
Baby sex, n (%)										
Male	31	50.80%	64	47.80%		27	43.50%	68	51.90%	
Female	30	49.20%	69	52.20%	0.85	35	56.50%	63	48.10%	0.35

Table 5.1. (cont'd)

Maternal age (year), mean (SD)	30.7	5.26	31	5.42	0.7	29.9	5.9	30.1	5	0.1
Birth weight (gram), mean (SD)	3251	620	3398	562	0.12	3244	587	3402	579	0.08
Gestational age (week), mean (SD)	38.3	2.1	38.6	1.8	0.28	38.2	2.2	38.7	1.7	0.14
Age at sleeping disturbance assessment	24.3	1.57	24.1	0.91	0.16	24.1	1.27	24.2	1.11	0.72

[1]The difference between sleep disorders and characteristics was analyzed by Chi-square test or One-Way Analysis of Variance (ANOVA).

Infancy fecal samples from children who have difficulty initiating sleep displayed a higher Shannon index (p-adjusted= 0.07) and a higher Chao 1 index (p-adjusted= 0.09) than samples from those who did not have difficulty initiating sleep (Figure 5.1A-5.1B). Infancy fecal samples from children who have difficulty maintaining sleep displayed a higher Shannon index (p-adjust= 0.004) and a higher Chao 1 index (p-adjust= 0.0004) than samples from those who did not have difficulty maintaining sleep (Figure 5.1C-5.1D). The multivariate model showed that children who had a higher Shannon index (OR = 2.4, CI:1.2-4.9, p-adjust = 0.04) and a higher Chao 1 index (OR = 1.01, CI:1.0-1.03, p-adjust = 0.008) at early infancy were more likely to have difficulty maintaining sleep (Table 5.2). There was also a non-significant trend towards an increased Shannon index (OR = 1.8, CI:0.95-3.6, p-adjust = 0.32) and an increased Chao 1 index

(OR = 1.1, CI:0.99-1.01, p-adjust = 0.64) being associated with a higher risk of having difficulty initiating sleep (Table 5.2).

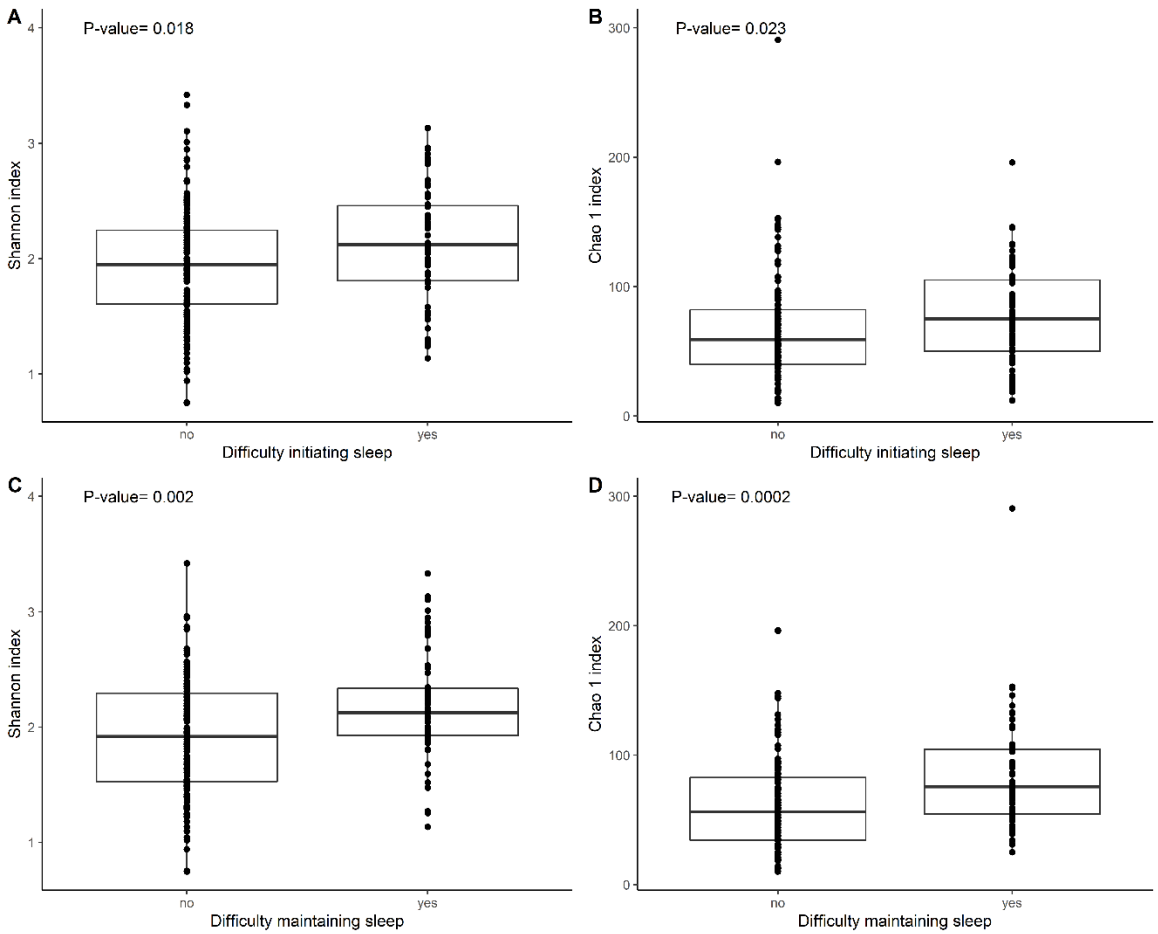

Figure 5.1. Boxplot of the alpha diversity (Shannon and Chao 1) by difficulty initiating and maintaining sleep. (A) Association between difficulty initiating sleep and Shannon index. P-adjust = 0.07 after Bonferroni correction. (B) Association between difficulty initiating sleep and Chao 1 index. P-adjust = 0.09 after Bonferroni correction. (C) Association between difficulty maintaining sleep and Shannon index. P-adjust = 0.008 after Bonferroni correction. (D) Association between difficulty maintaining sleep and Chao 1 index. P-adjust = 0.0008 after Bonferroni correction.

Table 5.2. Association between alpha diversity and difficulty initiating and maintaining sleep

	Shannon index			Chao1 index		
	Odds ratio (95% CI)	p-value	adjusted p-value[1]	Odds ratio (95% CI)	p-value	adjusted p-value
Difficulty initiating sleep	1.83 (0.95, 3.64)	0.08	0.32	1.05 (0.99, 1.01)	0.16	0.64
Difficulty maintaining sleep	2.41 (1.23, 4.93)	0.01	0.04	1.01 (1.00, 1.03)	0.002	0.008

P-value was adjusted by Bonferroni correction. All models were adjusted for breastfeeding status, delivery mode, birth weight, gestational age, age at fecal sample collection, maternal education level, pre-pregnancy BMI, race, baby sex, and maternal age

When classified by difficulty initiating sleep, the gut microbiota communities of the children were significantly differed based on unweighted UniFrac distance metric (PERMANOVA: R^2= 0.9%, p-value =0.04, Figure 5.2B), but were similar when compared based on Weighted UniFrac distance metric (PERMANOVA: R^2= 0.4%, p-value =0.57, Figure 5.2A). When classified by difficulty maintaining sleep, the gut microbiota communities were significantly differed based on the unweighted UniFrac distance metric (PERMANOVA: 1.8%, p-value =0.004, Figure 5.2D), but were not distinct based on the weighted UniFrac distance metric (PERMANOVA: R^2= 0.9%, p-value =0.1, Figure 5.2C).

We then performed differential abundance testing with DESeq2 to see which taxa were associated with the sleep problems. At the genus level, we detected a higher abundance of *Desulfovibrio, Butyricimonas, Roseburia,* and a lower abundance of the *Lachnospiraceae* NK4A136 group, *Collinsella* in the children with difficulty initiating sleep (q-value < 0.001, Table 5.3). We also identified a higher abundance of *Eubacterium coprostanoligenes* group, *Epulopiscium, Colidextribacter, Lactobacillus, Megamonas, Faecalibacterium, Roseburia,* and a lower abundance of *Lachnospiraceae* uncultured in the children with difficulty maintaining sleep (q-value < 0.001, Table 5.4).

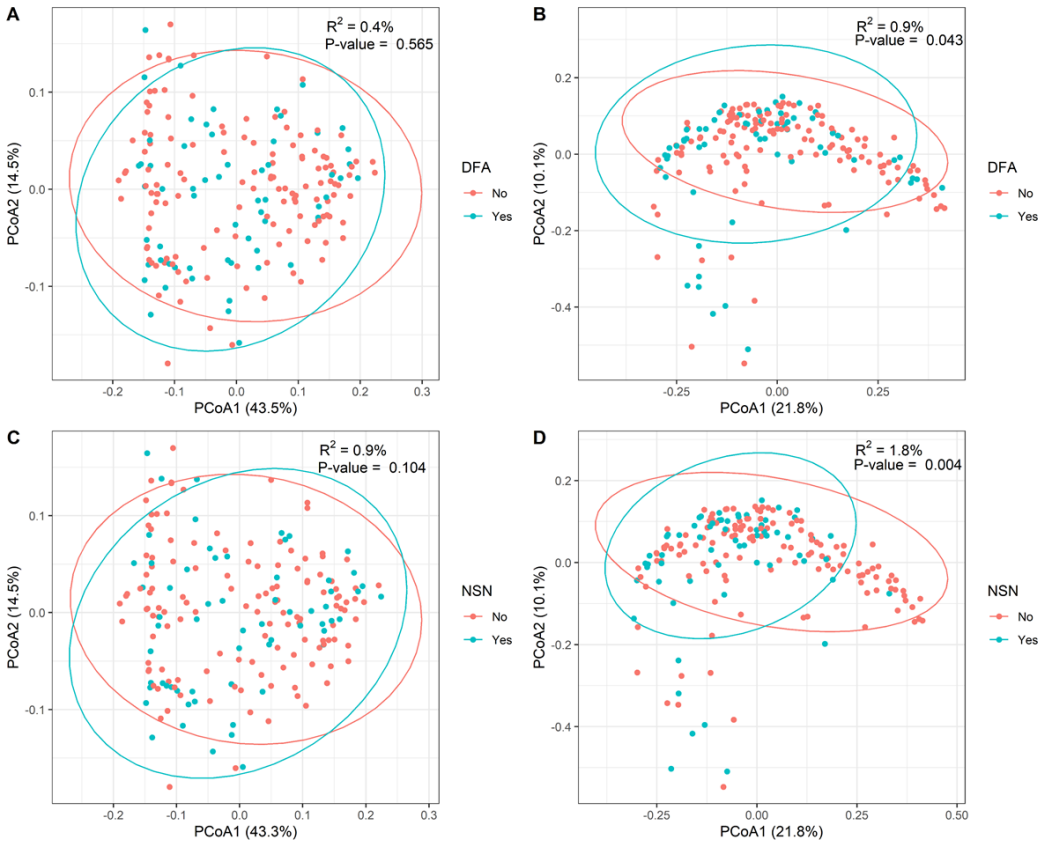

Figure 5.2. Principal Coordinates Analysis (PCoA) for difficulty initiating and maintaining sleep. R^2 and p-value were calculated by the PERMANOVA test. (A) PCoA for difficulty initiating sleep based on weighted UniFrac distance metric. (B) PCoA for difficulty initiating sleep based on unweighted UniFrac distance metric. (C) PCoA for difficulty maintaining sleep based on weighted UniFrac distance metric. (D) PCoA for difficulty maintaining sleep based on unweighted UniFrac distance metric.

Table 5.3. DESeq2 Analysis Results: Associations of gut microbiome taxa at genus level and difficulty initiating sleep adjusted by covariates[1]

Family	Genus	Log 2 folder change	p-value	p-adjusted
Desulfovibrionaceae	*Desulfovibrio*	1.32	7.48×10^{-8}	8.37×10^{-6}
Marinifilaceae	*Butyricimonas*	1.30	1.52×10^{-6}	5.67×10^{-5}
Lachnospiraceae	*NK4A136* group	-1.94	1.20×10^{-6}	5.67×10^{-5}
Coriobacteriaceae	*Collinsella*	-1.58	3.62×10^{-6}	1.02×10^{-4}
Lachnospiraceae	*Roseburia*	1.45	3.97×10^{-5}	8.90×10^{-4}

[1]All models were adjusted for breastfeeding status, delivery mode, birth weight, gestational age, age at fecal sample collection, maternal education level, pre-pregnancy BMI, race, baby sex, and maternal age. P-adjusted is the FDR (Benjamini-Hochberg) adjusted p-value. p-adjusted < 0.001 for multiple comparisons was considered statistically significant and included in the table.

Table 5.4. DESeq2 Analysis Results: Associations of gut microbiome taxa at genus level and difficulty maintaining sleep adjusted by covariates[1]

Family	Genus	Log 2 folder change	p-value	Adjusted p-value
Eubacterium coprostanoligenes group	Eubacterium coprostanoligenes group	1.81	3.32×10^{-9}	3.56×10^{-7}
Lachnospiraceae	*Epulopiscium*	1.98	3.09×10^{-7}	3.56×10^{-7}
Oscillospiraceae	*Colidextribacter*	1.98	3.48×10^{-7}	1.24×10^{-5}
Lachnospiraceae	*Lachnospiraceae* uncultured	-2.29	6.12×10^{-7}	1.64×10^{-5}
Lactobacillaceae	*Lactobacillus*	1.85	1.11×10^{-6}	2.38×10^{-5}
Selenomonadaceae	*Megamonas*	1.50	1.32×10^{-5}	2.2×10^{-4}
Ruminococcaceae	*Faecalibacterium*	1.41	1.44×10^{-5}	2.2×10^{-4}
Lachnospiraceae	*Roseburia*	1.39	7.14×10^{-5}	9.55×10^{-4}

[1]All models were adjusted for breastfeeding status, delivery mode, birth weight, gestational age, age at fecal sample collection, maternal education level, pre-pregnancy BMI, race, baby sex, and maternal age. P-adjusted is the FDR (Benjamini-Hochberg) adjusted p-value. p-adjusted < 0.001 for multiple comparisons was considered statistically significant and included in the table.

5.5 Discussion

Our prospective study demonstrated that the composition of the gut microbiome at early infancy was significantly associated with difficulty initiating sleep and maintaining sleep at the age of 2 years. We also identified those microbial candidates that might contribute to both sleep disorders. Our results suggested a clear association between infant gut microbiota composition and difficulty initiating sleep and maintaining sleep.

Due to different inclusion criteria, prevalence estimates of insomnia symptoms in childhood have varied from 4–41%[35–39]. In the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-4), a patient with difficulty initiating or maintaining sleep for at least one month was considered having a risk for insomnia. In our study, nearly half of the population had difficulty initiating sleep or maintaining sleep, but our survey did not ask for the of these symptoms. The univariate analysis showed that difficulty initiating sleep was significantly associated with maternal pre-pregnancy BMI. The multivariate analysis confirmed this finding that children whose mothers were overweight were more likely to have difficulty initiating and maintaining sleep. This association could possibly be mediated by child obesity, which was shown to be significantly associated with maternal pre-pregnancy BMI in many studies. Obesity, which is a risk factor for sleep disorders of all ages, has been linked to short sleep duration and poor sleep quality during childhood[40]. However, child BMI was not recorded in our study.

The univariate analysis also showed that children with difficulty maintaining sleep were more likely to be never fed with breast milk, although this association disappeared in the multivariate analysis with the odds ratio decreasing from 3.1 to 1.7.

Breastfeeding was found in other studies to be associated with increased night waking and sleep fragmentation at infancy due to parenting practices of nursing at night[41–43]. However, this

association disappeared by the 9-month follow-up[41,44], suggesting that sleep disruptions associated with breastfeeding resolve when the breastfeeding ends.

Turning to the microbiome findings, our results demonstrated that alpha diversity at 3 months of age predicted sleep disorder at 2 years of age. Higher Shannon index and Chao 1 index were significantly associated with a higher risk of difficulty maintaining sleep after adjustment of covariates. There was also a trend of increased Shannon index and Chao 1 index in the children with difficulty initiating sleep. The alpha diversity of gut microbiota is dynamic during infancy and is correlated with complementary feeding and delivery mode. High alpha diversity in infancy indicates a more mature, adult-like community[65]. Carlson et al. demonstrated that high alpha diversity at infancy was associated with poor cognitive development at 2 years of age[45]. However, numerous studies have shown that low alpha diversity in infancy is associated with asthma and type 1 diabetes[66-67]. A highly diverse microbiome can introduce functional redundancy[46], which allows individuals to adapt to environmental fluctuations, maintain intestinal homeostasis and support human health. Our results, together with the work done by Carlson et al.[45] suggest that increased alpha diversity at early infancy could contribute to sleep problems in childhood.

We noted that the structure of the infant gut microbiota affected difficulty initiating and maintaining sleep when the unweighted UniFrac distance metric was used. An unweighted UniFrac distance considers only species presence and absence information and counts the fraction of branch length unique to either community, while a weighted UniFrac distance uses species abundance information and weights the branch length with abundance difference[47,48]. Thus, unweighted UniFrac distance is most efficient in detecting abundance change in rare taxa.

Our results suggested that the association between gut microbiota composition and sleep disorders was potentially driven by rare and less abundant taxa[49,50].

Our results indicated that several taxa were positively or negatively associated with infant sleep problems, suggesting different taxa were involved in the mechanisms that influence the sleep disorders in children. Our results agree with the previous literature, which suggests that the genera *Desulfovibrio* and *Lactobacillus* were significantly enriched in the gut microbiome of insomniac adult participants[51,52]. An animal study also confirmed that *Desulfovibrio* could increase the risk of obstructive sleep apnea (OSA) by causing intermittent hypoxia during sleep[53]. Many studies have also confirmed that a higher abundance of *Desulfovibrio* is associated with the incidence and the severity of autism[54,55]. *Desulfovibrio* is a sulfate-reducing bacterium in the human gut that can generate hydrogen sulfide, which is an effective inhibitor of the oxidation of short-chain fatty acids (SCFAs) in cells[56,57]. Several species in the *Lactobacillus* genus were found to have beneficial effects on sleep rhythms in animal models, which is in contradiction to our results[58,59]. However, some *Lactobacillus* species can produce GABA, and abnormal expression of GABA mRNA is linked to depression and insomnia[60,61]. Thus, more studies are needed at the species level of *Lactobacillus* to understand the mechanism of the influence of gut *Lactobacillus* on sleep disorders.

An important limitation of this study is that only one stool sample collected. Gut microbial ecology and function are dynamic across the infancy lifestage by time and influenced by multiple factors, including feeding practice, delivery mode, and environmental exposures[62–64]. Future studies would benefit from a longitudinal stool sample collection during infancy that helps us better understand the association between infant gut microbiome and sleep disorders. Another limitation is that 16S rRNA sequencing can provide limited strain-level information. Previous studies

demonstrate a complex mechanism for several species of *Lactobacillus* in sleep disorders[60]. Thus, Shotgun metagenomic sequencing is needed for future studies.

APPENDIX

Table S5.1. Association between maternal/infant characteristics and difficulty falling sleep in infants[1]

	Odds ratio (95% CI)	P-value
Delivery mode, n (%)		
Vaginal delivery	ref	
C-section	1.94 (0.91,4.15)	0.08
Maternal education level, n (%)		
Did not finish high school	ref	
High school graduate or GED	0.55 (0.10, 3.18)	0.49
Some college	0.87 (0.18, 4.73)	0.87
College graduate or more	0.85 (0.17, 4.64)	0.84
Maternal pre-pregnancy BMI		
Normal	ref	
Overweight	3.73 (1.64, 8.72)	0.002*
Obesity	0.85 (0.35, 2.03)	0.72
Race, n (%)		
White	ref	
Black	1.08(0.34, 3.4)	0.89
Other	1.22(0.29, 4.48)	0.77
Breastfeeding status at fecal sample collection, n (%)		
Exclusive breastfeeding	ref	
Partial breastfeeding	0.87(0.33, 2.22)	0.78
Not breastfeeding	0.89(0.33, 2.33)	0.82
Ever breastfeeding, n (%)		
Yes	ref	
No	0.85(0.22, 3.15)	0.81
Baby sex, n (%)		
Male	ref	
Female	1.11(0.56, 2.23)	0.77
Maternal age (year)	0.98(0.91, 1.05)	0.62
Birth weight (kilogram)	0.41(0.17, 0.94)	0.04*
Gestational age (week)	1.13(0.89, 1.46)	0.32

[1]All variables listed in table were included in the multivariable model.

*<0.05

Table S5.2. Association between maternal/infant characteristics and difficulty maintaining sleep in infants[1]

	Odds ratio (95% CI)	P-value
Delivery mode, n (%)		
Vaginal delivery	ref	
C-section	1.5(0.71, 3.15)	0.29
Maternal education level, n (%)		
Did not finish high school	ref	
High school graduate or GED	0.52(0.11, 2.39)	0.40
Some college	0.69(0.16, 3.0)	0.62
College graduate or more	0.51(0.11, 2.28)	0.37
Maternal pre-pregnancy BMI		
Normal	ref	
Overweight	2.69(1.15, 6.39)	0.02*
Obesity	1.49(0.64, 3.48)	0.35
Race, n (%)		
White	ref	
Black	0.87(0.29, 2.57)	0.81
Other	2.1(0.54, 7.74)	0.26
Breastfeeding status at fecal sample collection, n (%)		
Exclusive breastfeeding	ref	
Partial breastfeeding	0.92(0.35, 2.33)	0.86
Not breastfeeding	0.99(0.38, 2.49)	0.98
Ever breastfeeding, n (%)		
Yes	ref	
No	1.72(0.51, 5.81)	0.38
Baby sex, n (%)		
Male	ref	
Female	1.64(0.83, 3.29)	0.16
Maternal age (year)	0.97(0.9, 1.04)	0.35
Birth weight (kilogram)	0.63(0.27, 1.38)	0.26
Gestational age (week)	1.01(0.80, 1.28)	0.93

[1]All variables listed in table were included in the multivariable model.

*<0.05

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CHAPTER 6. SUMMARY, LIMITATIONS AND FUTURE RESEARCH

Our studies examined whether feeding practices affect early gut microbial colonization and whether gut microbial colonization influence temperament and sleep disorders in children using a sample of singleton births from an ongoing population-based pregnancy and birth cohort set in Michigan's lower peninsula. In this prospective study, fecal samples were collected between 3-9 months, and the bacterial 16S rRNA gene (V4 hypervariable regions) was amplified. We then collected temperament and sleep outcomes of infants at 9 months and 2 years, respectively.

In the first analysis, we confirmed the importance of breastfeeding and vitamin D supplements in shaping gut microbiota composition in early infancy. By performing differential abundance analysis on individual taxa, our results demonstrated that vitamin D supplement potentially reduced the likelihood of *Haemophilus* colonization in exclusively breastfed infants. The Vitamin D family is a group of fat-soluble secosteroids absorbed from sunlight, food or supplements. Vitamin D exists in several forms, including ergocalciferol (D2), cholecalciferol (D3), 1-hydroxylated and 1,25-hydroxylated forms[12-13]. In addition to the food, humans acquire vitamin D synthesized in the skin upon exposure to sunlight. vitamin D synthesis is affected by season, time of day, skin pigmentation, cloth, aging etc.[13]. The liver and kidneys convert vitamin D into 25-hydroxyvitamin D which is a major circulating form and 1,25-dihydroxyvitamin D which is the biologically active form, respectively[13-14]. Vitamin D is essential for bone development and calcium homeostasis[15]. In addition, vitamin D may play a critical role in immune regulation, partially via modulating the gut microbial composition[16]. Vitamin D deficiency is associated with dysbiosis that promotes inflammation[16]. Our results confirm and extend existing findings and point out that vitamin D potentially protects against *Haemophilus,* which can cause a wide variety of infections in infants. However, only 39.8% of the exclusively breastfed infants received vitamin D supplements in our study population. Our

results highlight the need for actions to support the promotion of vitamin D among exclusive infants.

In the analysis of the next two aims, we focused on temperament and sleep disorders which are two critical outcomes closely related to brain development during childhood. Uncovering the relationship between gut microbiota and these two outcomes will help us better understand the operation of the microbiota-gut-brain axis. Our results demonstrated an association between gut microbiota composition and the temperament characteristic described as negative emotionality. Negative emotionality, defined as a disposition to experience negative emotions such as anger and fear[1], is one of the most important dimensions of infant temperament. An excess of negative emotionality may contribute to a high risk of later childhood psychopathology and behavior problems[2]. Thus, the present study provides further evidence of the critical functional roles of the gut microbiota in behavior problems. In a sensitivity analysis, we discovered a potential role of infant vitamin D intake in modifying the association between microbiota clusters and negative emotionality. The association described above was identified only among the infants who were not recipients of vitamin D supplements, suggesting that infant vitamin D intake potentially protects against the adverse effect of the gut microbiome–associated on infant emotionality. Finally, we demonstrated that the composition of the gut microbiome in early infancy was significantly associated with difficulty initiating sleep and maintaining sleep at the age of 2 years. Epidemiologic studies indicate that up to 50% of children have experienced a sleep problem by age six[3]. A growing body of evidence shows that sleep disorders and insomnia in early childhood may be linked obesity, diabetes, and inflammatory diseases in later life[4–6].

Previous research has focused on understanding the psychological, social, and physiological factors that regulate sleep. Our study, together with recent studies, provides a new basis for understanding the mechanisms of sleep problems. Circadian Rhythm, which is an important process in the regulation of sleep, has been linked to gut microbiota in recent studies[7]. Studies have demonstrated that the gut microbiota exhibit compositional and functional rhythmic fluctuations[8]. Consequently, the intestinal epithelium is exposed to different bacterial species and their metabolites throughout the day[8]. In turn, the circadian rhythm of the microbiota regulates the transcription of host circadian clock genes and affects epigenetic modifications and oscillations in metabolite levels[8,9].

These results add to the mounting evidence connecting the gut microbiota with the gut-brain axis, where early gut microbial colonization may be linked with neurodevelopmental outcomes with potential long-term effects. Within this axis, gut microbiota affects brain function through various pathways, including the immunoregulatory pathway, neuroendocrine pathway, vagus nerve pathway, and tryptophan metabolism[10]. The term psychobiotic was brought up by Dinan et al.[11], as a novel class of psychotropic medication, defined as a "live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness." Research into the microbiota-gut-brain axis could help us develop potential new therapeutic targets for the effective treatment or prevention of brain disorders.

One limitation of this dissertation is that only one fecal sample was included in our analysis, and infant gut microbiota matures rapidly maturated the first year of life. Thus, our results may not fully represent the temporal development of the infant gut microbiome, which may be critical to studying neurodevelopment in early life. A second limitation is that 16S rRNA sequencing can only provide limited strain-level information. Previous studies have demonstrated the biological

importance and different metabolic capabilities of specific bacterial strains on human health. A third limitation is that due to the small sample size and lack of power, we are unable to assess the potential role of gut microbiota in the causal pathway linking infant feeding practices and brain development.

Future studies using this same cohort may be able to extend our study in four ways. First, we may use shotgun metagenomic sequencing, which provides higher resolution and sensitivity in microbiome analysis. Metagenomics also allows us to access the species-level functional profiling of the gut microbiota, which is important for understanding gene and metabolic pathway content. Second, vitamin D, which is an important factor for gut microbiota, has only been studied in vitamin D supplements for infants in our study. However, previous studies highlight the influence of prenatal vitamin D and vitamin D concentration in breast milk on infant health. We may be able to estimate prenatal vitamin D intake by interview and to measure vitamin D concentration in mother's breast milk and in archived prenatal serum. Third, we can use Mendelian randomization to make casual inferences for our existing findings. Mendelian randomization is a method of using measured variation in genes of known function to examine the causal effect of a modifiable exposure on disease in observational studies. Fourth, as the sample size of our cohort increases, we may be able to assess the potential role of gut microbiota in the causal pathway linking infant feeding practices and brain development.

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