Secondary Analysis on the Impact of Sleep-Wake Patterns and Feeding Practices in the Gut Microbiome in the First Years of Life Trailed into Toddlerhood

by

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ABSTRACT

The second an individual is born, the gut microbiome starts acquiring unique characteristics, including microbial richness and evenness. In addition, it has been found that during infancy, the mode of delivery, antibiotic exposure, feeding patterns, and environment play a role in such development. However, infancy is still an understudied population related to the gut microbiota, specifically its' connection to two modifiable factors-sleep-wake patterns and its' interconnection with feeding practices in the first year of life. This secondary data analysis from a randomized longitudinal intervention study assessed the efficacy of a home-based education program in preventing the onset of childhood obesity.157 A convenience sample of 40 Hispanic mother and infant dyads were recruited to participate in an additional collection of fecal samples to evaluate associations between lifestyle/behavioral factors in infancy and gut microbiome composition in toddlerhood. Total sleep duration and feeding practices (breastmilk and formula) were assessed at one, six, and twelve months. In addition, alpha and beta diversity metrics were assessed from infant stool samples collected at 36 months. This study found some significant and trending values for pairwise comparisons of alpha (Shannon Diversity Index) and beta (Bray Curtis and Jaccard Distance). Sleep-wake adequacy consisted of 14-17 total hours of sleep in 24 hours at one month and 12-16 hours for six and twelve months of age. No significant values were identified at one month of age. However, six and twelve months demonstrated significant observations for gut microbial richness and evenness. Trending differences (p=.06, Shannon Diversity Index) persisted in infants receiving adequate sleep at six months but for different feeding modalities. Faith's PD, Pielou's measure, and observed OTUs were three additional alpha-diversity metrics performed in all groups. Observed OTUs (p=.03) and Faith's PD (p=.03) were significant at twelve months, demonstrating an increased microbial feature in infants receiving adequate sleep. The findings from this study may show how different timestamps in the first year of life may create gut microbial milestones. However, the interrelation between sleep-wake patterns, feeding modalities, and gut microbiome development is limited; further investigation is needed to monitor close changes and potentially create a criterion

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INTRODUCTION

In the United States, approximately 4 out of 10 adults are obese(CDC, 2022b) and about 20% of children and adolescents (aged 2-19 years) carry excess weight for their age.(*Childhood Obesity Facts | Overweight & Obesity | CDC*, 2022) Despite prevention efforts, obesity continues to impact younger children (*Prevalence of Obesity and Trends in Body Mass Index Among US Children and Adolescents, 1999-2010 | Adolescent Medicine | JAMA | JAMA Network*, n.d.)*,*(*Read "Early Childhood Obesity Prevention Policies" at NAP.Edu*, n.d.); as 10% of infants and toddlers carry excess weight(*Read "Early Childhood Obesity Prevention Policies" at NAP.Edu*, n.d.). Of those under 2 years, 8.1% are at or above the 95th percentile, according to CDC growth charts(Ogden et al., 2014). There has been an increased awareness of obesity since it was observed to be one of the key determining factors for the development of life-threatening conditions such as diabetes, uncontrolled cardiac issues, cancers, liver and kidney diseases, among others.[3] These cardiometabolic conditions are also amongst the most expensive to medically treat, equating to approximately $190 billion dollars annually in the United States.(*Economic Costs of Obesity | Healthy Communities for a Healthy Future*, n.d.)

The prevalence of obesity doesn't instantaneously spark in adulthood, as almost half of overweight adults had a history of carrying excess weight as children(Serdula et al., 1993). In fact, childhood obesity is responsible for $14 billion in direct medical costs from the 21% of annual medical spending in the United States for excess weight gain-related illnesses(*Economic Costs of Obesity | Healthy Communities for a Healthy Future*, n.d.). Despite robust data that account for numerous factors that play a fundamental role in the onset of obesity from birth to childhood, very few studies have focused on determinants of rapid weight gain in the first year of life(*Protocol of the Snuggle Bug/Acurrucadito Study: A Longitudinal Study Investigating the Influences of Sleep-Wake Patterns and Gut Microbiome Development in Infancy on Rapid Weight Gain, an Early Risk Factor for Obesity | BMC Pediatrics | Full Text*, n.d.).

The theory of developmental origins of health and disease is based on the concept that environmental factors acting in early life have a profound effect on increasing the vulnerability of an individual to be affected by a chronic disease later in life.(Gluckman et al., 2010) This intense period of growth and development is gaining attention for social, behavioral, and environmental risks for obesity and cardiometabolic diseases. Rapid weight gain in infancy may be an important precursor to the development of uncontrolled weight management that trails into adulthood(Serdula et al., 1993). RWG during infancy is defined as a greater than 0.67 positive change in weight-for-age Z-score (on standard growth charts) across time.[8]

Before addressing determinants that increase excess weight gain in infants; understanding how fixed and modifiable factors influence the gut microbiome’s role in rapid weight gain in the early stages of life is of high priority.(*Determinants and Duration of Impact of Early Gut Bacterial Colonization - FullText - Annals of Nutrition and Metabolism 2017, Vol. 70, No. 3 - Karger Publishers*, n.d.) An individual becomes a host to a wide array of microbes the second he/she is born. Beyond mode of delivery, other early-life events influence microbial composition including mother-infant bond, feeding mode, latch if breastfeeding, sanitation of the environment, and medication use. While all of this is happening, the human intestine is rapidly being influenced by such and begins to start being colonized by an array of microbes.[18] The gut then begins to become host to a colony of a vast number of bacteria and microorganisms; this microbial community is defined as the gut microbiota/microbiome.[19] A global overview of the relative abundances and initial microbes that develop in the gastrointestinal tract postpartum include Proteobacteria, Fusobacteria and Bacteroidetes.[20] Microbes such as these play essential duties and assist a newborn to digest foods, absorb nutrients and start building their immune system. While these processes are occurring, the gut microbiota starts acquiring characteristics that have been found to be involved in the control of body weight, energy regulation, and inflammation(Sanchez et al., 2015).

Breastmilk is considered the normative standard for infant feeding, and is believed to provide optimal nutrition in early life.[9] Multiple studies highlight the vast nutritional benefits this feeding mode provides; such as optimal macro/micro-nutrient delivery,[10] bioactive components that have an impact on body function,[11] and microbiome structure development(Stewart et al., 2018). Exclusive, demand-based breastfeeding has been recommended as the optimal nutrition for the first six months of life, continued by meeting half of a child’s nutritional needs throughout the second half of their first year.[24] However, this is not the main form of nutrition for every child which may possibly influence their growth and development. It is important to recognize that even though it is recommended that all infants are exclusively breastfed for the first six months of life,(SECTION ON BREASTFEEDING et al., 2012a) only one in four infants meet this. This opens the discussion of the physiological effects of formula use and whether different trends in growth and weight are observed between breastfed vs. formula-fed infants during their first year of life.

Formula-derived nutrition is essential when breastmilk is not a viable nutritional resource for an infant, however, future analysis is needed to differentiate early rapid weight gain trends when comparing formula-fed infants and breastfed infants; since not many studies have shown how this may have significant effects on early rapid weight gain.(Mennella et al., 2019) (Dewey, 1998) However, research has begun to demonstrate possible significant differences in formula-fed and breast-fed infants. A study of 97 infants reported a 43% rate of rapid weight gain percentages regardless of feeding mode but also suggested that formula-fed infants with rapid weight gain had increased odds for becoming overweight at one year while breastfed infants with rapid weight gain, did not.(*Impact of Early Rapid Weight Gain on Odds for Overweight at One Year Differs between Breastfed and Formula-fed Infants - Trabulsi - 2020 - Pediatric Obesity - Wiley Online Library*, n.d.) Another possible factor to discuss in further research is the quality of the breastmilk and formula infants receive. A study published in The American Journal of Nutrition observed increased weight velocities in infants receiving cow milk formula when compared to infants who were randomly assigned to receive extensively protein hydrolyzed formula(*Type of Infant Formula Increases Early Weight Gain and Impacts Energy Balance: A Randomized Controlled Trial | The American Journal of Clinical Nutrition | Oxford Academic*, n.d.), also derived from cow's milk.

There are established and highly studied determinants of rapid weight gain during infancy; such formula vs breastfeeding practices (as discussed above), socioeconomic status, and timing of introduction to solid foods.(Huh et al., 2011) Sleep-wake rhythms may also influence nutrition and weight trajectories.(Crispim et al., 2011) Studies performed in adults show how the reduction of sleep may have an influence on metabolic and endocrine processes that are concluded to be major risk factors related to the development of obesity. Less is known about infants but researchers are beginning to explore sleep-related behaviors during this formative period. A study conducted by Petrov et al. analyzing infant nap frequency in combination with nocturnal vs diurnal sleep distribution found that one-month-old infants who are napping five times or more per day had significant associations with decreased odds for RWG(Petrov et al., 2021). Also, findings have given the rise to address how different feeding practices affect the routine of an infant during their first year of life, specifically weight and growth comparisons and sleep schedule.(Mennella et al., 2019) (Dewey, 1998) Mothers who exclusively breastfeed also have significant differences of nocturnal sleep compared to non-exclusive feeding providers.(Doan et al., 2014) The National Sleep Foundation has established daily sleep duration recommendations. For instance, newborns aged 0-3 months require 14-17 hours , while 12-15 hours are recommended for infants aged 4-11 months and 11-14 hours for toddlers 1-2 years old.[16] Sleep guidelines established by the NSF are based on methods and practices established by panels of experts who develop consensus recommendations for the population based on the most current research evidence and systematic literature reviews.("Guidelines," n.d.) Data indicate that short sleep duration, irregular sleep-wake patterns, and inadequate sleep quality are found to be associated with weight gain as well as "obesogenic behaviors" in children and adolescents(Lebenthal & Tauman, 2021). However, such a link has not been yet to be profoundly studied/analyzed in the earlier years of life.

Purpose of Study

The purpose of this secondary analysis is to explore how changes in sleep duration from one month of age up to one year of life impact the gut microbial diversity of toddlers. This study will also assess how sleep-wake patterns interact with feeding modes (breastfed vs. formula fed) during an infant's first year of life during specific time periods (1, 6, and 12 months) and contribute to the gut microbiome diversity and community structure at 3 years of age.

Research Aims and Hypothesis

Aim 1: Explore associations between early sleep-wake (as well as feeding events) in infancy and alpha diversity of the gut microbiome (microbial richness measured via Shannon's Index) at three years of age**.**

H1: Infants meeting total daily sleep requirements at one, six, and twelve months, will have greater gut microbial diversity in toddlerhood than those not meeting requirements.

H2: Infants receiving breastmilk and meeting total daily sleep requirements at one, six, and twelve months, will have greater gut microbial diversity in toddlerhood compared to infants that are formula-fed and/or are not meeting total sleep recommendations.

Aim 2: Explore associations between early sleep-wake (as well as feeding events) in infancy and beta diversity of the gut microbiome (microbial variance measured using Bray Curtis and Jaccard distances) at three years of age**.**

H1: Infants meeting total daily sleep requirements at one, six, and twelve months, will have greater gut microbial community variance in toddlerhood then those not meeting requirements.

H2: Infants receiving breastmilk and meeting total daily sleep requirements at one, six, and twelve months, will have greater gut microbial community variance in toddlerhood compared to infants that are formula-fed and/or are not meeting total sleep recommendations.

CHAPTER 2

REVIEW OF LITERATURE

Prevalence of Obesity

Obesity has received significant attention due to its exacerbating effect on the nation’s health status. The CDC reports that around 40% of the population in the United States falls into the obese category.(*Products - Data Briefs - Number 360 - February 2020*, 2020) In addition to the detrimental effect on an individual's quality of life due to the domino effect of obesity in the body, the cost of managing obesity and related diseases is around $173 billion per year nationwide.(Ward et al., 2021) Adults are not the only ones being affected by obesity; younger generations also experience overweight and obesity.

Childhood Obesity

Early childhood obesity is a health concern affecting approximately 14.4 million children and adolescents. Rates of childhood obesity have been increasing over the years, and there is more than one singular factor to account for such a rise. From a young age, the likelihood of developing obesity is highly impacted by the surroundings of an infant. For example, statistics show that if one parent is obese, the child has a 50% chance of being obese, and if both parents are classified as obese, there is an 80% chance that the infant in that setting will develop obesity.(AACAP, n.d.) Weight status for a child is classified differently from adults for who body mass index is used. In infants aged two or younger, weight-for-length percentiles established by the World Health Organization (WHO) are used to assess growth trends.(*Infant Growth Chart Calculator: Weight Length WHO 0-2 Year*, n.d.) In all children aged older than two. The growth charts universally adopted by pediatric practices are length-for-age and weight/BMI-for-age percentiles.(*Overview of the CDC Growth Charts*, n.d.) Nutrition status indicators for the CDC growth charts include overweight, at risk for overweight, underweight, and short stature. Infants and children falling into the equal to or higher to the 95th percentile (for both weights for length and weight/BMI for age) are categorized as overweight. In 1978("How Childhood Obesity Rates Have Changed Over Time," n.d.), only 5% of children were categorized as obese, and in 2018, the prevalence increased to 19.3%(*Childhood Obesity Facts | Overweight & Obesity | CDC*, 2022), equating to an increase of approximately 15% during the last forty years.

Specific Targeted Populations: Analyzing Obesity Statistics

Even though any individual may contribute to the increasing prevalence of obesity, it is evident that obesity and obesity-derived conditions affect some groups more than others. According to a CDC National Center for Health Statistics (NCHS) data brief, non-Hispanic Black adults (49.6%) had the highest age-adjusted prevalence of obesity, followed by Hispanic adults (44.8%)(*Products - Data Briefs - Number 360 - February 2020*, 2020). A similar pattern has also been observed for children. It is imperative to point out that obesity prevalence in children has increased despite their race. However, Hispanic, and Mexican American children are more likely to be overweight or obese. In the last survey period (2017-2018), 25.9% of Hispanic children and 26.9% of Mexican American children were obese, followed by 24.2% Hispanic-Black, 16.% Non-Hispanic White, and 8.7% of Non-Hispanic Asian(*Products - Health E Stats - Prevalence of Overweight, Obesity, and Severe Obesity Among Children and Adolescents Aged 2–19 Years,* 2021). With such high prevalence amongst these groups, many questions arise around questioning specific markers and confounding variables that increase the likelihood of carrying excess weight. There are multiple layers to uncover to determine what increases Hispanics' likelihood of developing obesity.

Etiology of Obesity and its' Downstream Effects

Excess Weight/Obesity Contributors

Over the last years, studies on variation of genetic factors influencing obesity have emerged.(Loos & Yeo, 2022) One study assessing genomic DNA data from 223 overweight and individuals with obesity (BMI of 25-45 kg/m2(Wu et al., 2010), identified in Hispanic children six single nucleotide polymorphisms (SNPs) as being associated with obesity. A clear pattern of a single gene variant (monogenic obesity) is rare, disputing genetics as leading contributing factor to obesity in targeted ethnic groups. There is insufficient data to conclude that there are specific markers that directly affect weight gain/predisposition to becoming obese. The majority of the research and data presented on specific contributing factors for the higher prevalence of Hispanics are in adults, and few studies have focused on children. Although most literature on social determinants of health and obesity amongst the Hispanic population is on adults, it is imperative to point out how it has been suggested that environmental factors (controlled or not controlled by adults) affect children's development and play a vital role in child's overall growth. (*Environmental Factors That Contribute to Child Vulnerability | Changing the Odds for Vulnerable Children : Building Opportunities and Resilience | OECD ILibrary*, n.d.) (Goldberg & Carlson, 2014) (Council (US) & Medicine (US), 2004) These children are placed in this environment from birth by their caregiver (s), granting no control over them and becoming non-modifiable factors for the child.

Social Determinants of Health in the Hispanic Population

Social determinants of health consist of five domains based on an individual's background, looking closely at where they were born, live, education, health care access, neighborhood, economic stability, and social/community context.(*Social Determinants of Health - Healthy People 2030 | Health.Gov*, n.d.) Some communities have a more resounding lack of equitable resources. The Hispanic population often faces such hardships. Obesity and health can be partly influenced by inadequate opportunities and resources including neighborhoods, education quality, safety in our communities, and even a clean environment with access to clean food and water(*Social Determinants of Health - Healthy People 2030 | Health.Gov*, n.d.). Javed et al. examined the correlation between Social Determinants of Health (SDOH) burden and overweight/obesity trends. Some ways to measure burden were by analyzing SDOH characteristics: difficulty/unable to pay medical bills, worry about economic stability (including paying monthly bills, retirements, medical costs), neighborhood, food insecurity, language proficiency, being uninsured, and delayed medical care, amongst others. (Javed et al., 2022)The results found that there was a tremendous increase in the burden of obesity with increased levels of social disadvantage; participants with the highest SDOH burden had approximately 15%, 50%, and 70% higher relative prevalence of overweight, obesity class 1 and 2, and obesity class 3, respectively, when compared to those with the lowest burden. (Javed et al., 2022)

Acculturation

Analyzing cultural backgrounds and the components of established cultural values is fundamental to understanding how obesity goes beyond individual choice; and how it may be "seeded" from a very young age. Research has found how acculturation plays a tremendous role in weight perception, food intake, diet quality, increased obesity rates, and the potential risk of developing secondary, weight-related diseases, specifically in Hispanics. Acculturation is defined as acquiring cultural elements of the dominant society into American culture/society(Lara et al., 2005). Associations between the country of birth and language usage, body/weight dissatisfaction, and weight loss intentions were performed in a study(New et al., 2013) using data from the National Health and Nutrition Examination Survey. The studied sample included 328 US-born and 347 foreign-born Hispanic obese adults, of which 23% used Spanish only, 51% used both Spanish and English, and 26% used only English. The results showed that extraordinary acculturation measures (defined by country of birth and language usage) had higher associations with weight dissatisfaction, weight loss intentions, and adequate weight perceptions. Obese Hispanics with lower language acculturation had lower likelihood of weight loss intentions and success if achieved. In addition, the findings showed that US-born obese Hispanics were more likely to perceive themselves as overweight and desire weight loss than their foreign-born counterparts. This suggests how Hispanic children may carry weight perceptions that may trail into adulthood based on their language usage.

Influence of Acculturation on Dietary Patterns

Food plays a fundamental role in society. Besides nourishing and giving energy to the body to perform day-to-day activities, it also connects individuals socially as they often sit together for meals. Research has shown how dietary patterns contribute to increased or decreased health risks. For example, positive changes in dietary intake, such as decreased saturated fat intake, have been shown to lower blood pressure.(Appel et al.,

1997) (“The Effects of Nonpharmacologic Interventions on Blood Pressure of Persons with High Normal Levels. Results of the Trials of Hypertension Prevention, Phase I,” 1992) Conversely, diets high in refined grains may affect the development of insulin resistance. (Hu et al., 2022) Acculturation plays a role in food choices and dietary behaviors. A study found that food choices in adults were impacted by cultural contexts and patterns, social structures and norms, resources/media influence, household/family structure, and nutrition transitions.(Haghighian Roudsari et al., 2017) Hispanics in the US have been shown to have a diversity of food traditions and eating practices which evolve as they are introduced to new foods in the US . (*Dietary Patterns of Hispanic Elders Are Associated with Acculturation and Obesity | The Journal of Nutrition | Oxford Academic*, n.d.)

Many research studies that analyze the obesogenic practices in Hispanics have been performed in adults. However, many of these adults can be potential heads of households and parents making them lead influencers in developing health behaviors and decision-makers for the younger generations in their homes. According to the 2015-2016 National Health and Nutrition Examination Survey, Hispanic/Latino children (ages 2-19 years old) in the United States had an obesity prevalence of approximately 25.8 % while 14.1% of non-Hispanic/Latino Whites are obese.(“National Obesity Monitor,” n.d.) This higher prevalence among Hispanic individuals continued into 2017-2018, with Hispanic children at 25.6%, followed by Non-Hispanic Black children at 24.2%, and Non-Hispanic Whites at 16.1%. High rates of obesity in the earlier years bring into question how obesogenic behaviors may also be occurring at an earlier age. Evidence suggests ethnic/racial differences in food-related parenting practices and their impact on increasing the risk for obesity.(Blissett & Bennett, 2013) Hispanic families' food parenting practices are somewhat understudied; however, the Parenting Strategies for Eating and Activity Scale (PEAS) Study(Sinan et al., 2019) examined how the food environment and sociodemographic characteristics were associated with obesogenic dietary intake among children.(LeCroy et al., 2019) The following parenting practices were assessed: Indulgent, Rules and Limits, Monitoring, and Pressure to Eat. Obesogenic intake measurement included the availability and consumption of snacks foods, sweets, and high-sugar beverages. It was found that parents with greater use of Rules and Limits, Monitoring, and Pressure to Eat styles had higher odds (OR: 1.75, 95% CI: 1.02, 3.03) of having children with obesogenic dietary intake vs. those with an indulgent parenting style. Children (12-16 years) of pressuring parents (High Pressure to Eat, Low Rules and Limits and Monitoring scores) also had 2.96 times greater odds of adopting obesogenic dietary behaviors. Further research is needed since the study concluded the need for further longitudinal studies examining associations of food feeding practices and dietary intake and its' impact on Hispanic youth.

Food Insecurity Status of U.S Households with Children

Among U.S households with children under eighteen, 14.8% of households with children are affected by food insecurity. For 322,000 households, one or more children experienced reduced food intake and disrupted eating patterns in 2020.(*USDA ERS - Key Statistics & Graphics*, n.d.) Food insecurity among adults is associated with obesity.(Pan et al., 2012) A lack of consistent access to food to fuel the body's energy needs does not only affect adults but also children. Arthur M. Lee et al(Lee et al., 2018). found that obesity was more prevalent among food-insecure children starting from kindergarten to third-grade students. Further interesting, is that among children the highest associations between greater body mass index and food insecurity were found in those with higher cortisol levels.(Distel et al., 2019)

Impact of Obesogenic Behaviors on Overall Health Across the Lifespan

People who carry excess weight (obesity is classified as a $\text{BMI} \geq 30 \text{ kg/m}^2$ are at risk of developing cardiometabolic conditions that negatively impact the quality of life compared to those with “healthy” weights(CDC, 2022c) (BMI: 18.gm-24.9 kg/m^2). These comorbidities include hypertension, high LDL cholesterol, Type 2 Diabetes (T2DM), renal diseases, sleep apnea, mental illnesses such as anxiety and clinical depression, types of cancer, and the leading cause of death for men and women: myocardial infarction. Heart disease alone is the cause of one in four deaths. The majority of the variables that heighten its' risk of occurrence are related to lifestyle-adopted behaviors such as physical inactivity and diets predominantly high in saturated fat. Besides heart disease, Diabetes is among the top ten leading causes of death in the United States(*FastStats*, 2022). According to the CDC, more than 37 million Americans have Diabetes, and approximately 90%-95% of them have Type 2 Diabetes.(CDC, 2022a) Some of the risk factors linked to increasing the likelihood of T2DM are related to lifestyle behaviors such a being overweight, low physical activity, and a history of Gestational Diabetes (if female).(*Diabetes Risk Factors | CDC*, n.d.) Although many obesity-related diseases have commonly affected adults, these health issues also extend to adolescents and children.(CDC, 2017)

Obesity in the formative years may result in cardiometabolic diseases(*Childhood Obesity and Cardiovascular Dysfunction - PubMed*, n.d.; *Childhood Obesity and Risk of the Adult Metabolic Syndrome: A Systematic Review - PubMed*, n.d.) (such as heart attack, insulin resistance(Bacha & Gidding, 2016), and non-alcoholic fatty liver disease(Africa et al., 2016)), high blood pressure, high cholesterol, respiratory conditions (such as asthma(Mohanan et al., 2014) and sleep apnea(Narang & Mathew, 2012)), and musculoskeletal disorders/discomfort, and gastrointestinal distress. Besides the development of physical-related health impediments, children with obesity can struggle with anxiety, depression, low self-esteem, and in some cases, face bullying and body stigma. (Beck, 2016; Halfon et al., 2013; Morrison et al., 2015)Also, rapid weight gain (RWF), defined as an increase in weight-for-age Z-score of 0.67 standard deviations at six months of age(*Childhood Obesity Facts | Overweight & Obesity | CDC*, 2022), has been established as a risk for obesity and trailing into childhood, adolescence, and adulthood(Ogden et al., 2014). In addition, its link to cardiovascular disease in adulthood can also be a concern for RWG since it has also been associated with arterial wall thickening and diastolic blood pressure.(*Childhood Obesity and Cardiovascular Dysfunction - PubMed*, n.d.)

Direct Modifiable Factors that Impact Weight During Infancy

The first one thousand days of life, defined as the period between conception and the second year of life, is considered a critical period that has short and long-term impacts on the health and well-being of the infant and the mother as well.(Hanson et al., 2015)(Koletzko et al., 2017) Unfortunately, non-modifiable factors that may increase the risk of health-related impediments in infants, such as biological and developmental genes, sociodemographic, household income, parent education, acculturation, and even community, do not allow for any control or direct interventions to modify an infant's quality of life from the moment they are introduced into the world. However, modifiable risk factors such as gestational weight gain, mode of delivery, caregiver's feeding practices, physical activity, and sleep duration(Hanson et al., 2015) may hold space for promising interventions to support an infant's adequate growth.

Feeding Practices

Feeding practices are a modifiable factor that may influence health outcomes in early life. Receiving nutrition via breastmilk or formula is one of the first interactions a newborn encounters. Feeding practices during such a critical period of child development is an ongoing topic of research interest(SECTION ON BREASTFEEDING et al., 2012a). Formula-fed infants have a significantly higher weight-for-age z-score than those who are exclusively breastfed. The World Health Organization(*Breastfeeding*, n.d.) recommends that infants be exclusively breastfed for the first six months of life. The Academy of Pediatrics(Section on Breastfeeding, 2012) and the Academy of Nutrition and Dietetics(Lessen & Kavanagh, 2015) recommended that it be complementary up until at least 12 months. The decision to breastfeed or not is highly influenced by many other factors, including complex pregnancy(Kozhimannil et al., 2014), early infant feeding, cultural traditions, and a supportive environment, especially in the hospital post-partum(Kozhimannil et al., 2014). Although infant formulas are intended to be an adequate substitute for breastmilk and mimic its nutritional composition, some differences are still found. Strict regulations ensure that all formulas include the proper amount of water, carbohydrate, protein, fat, vitamins, and minerals.(C. R. Martin et al.,

2016) There are variations in the types of formulas, ranging from the most common, bovine (cow)-based(Fiocchi et al., 2010), to soy(Cook, 1989), hypoallergenic(C. R. Martin et al., 2016) , and amino acid formulas(Cook, 1989) for those infants with severe milk and other allergies. Human breast milk's main components include proteins, lactose, lipids, sugars (carbohydrates), biologically active components, and various microbial communities speculated to colonize the infant's gastrointestinal tract.(Witkowska-Zimny & Kaminska-El-Hassan, 2017)

Breast milk is initially colostrum, rich in secretory immunoglobins, lactoferrin, and leukocytes to support epidermal growth,(Castellote et al., 2011) and transitions to fully mature milk after four to six weeks post-partum.(Kulski & Hartmann, 1981) A study published by the American Journal of Clinical Nutrition(Nommsen et al., 1991) examined how maternal characteristics and human breast milk macronutrient compositions four months post-partum can be associated with maternal body weight for height, protein intake, return of menstruation, and nursing frequency. Specific components of breastmilk depend on the mother's exposures and dietary consumption, such as macrophages, to protect against infection and T-cell activation(Agarwal et al., 2011) and micronutrient availability(Allen, 2012); however, the nutritional quality of human milk is still believed to be highly conserved.(Nommsen et al., 1991) Human milk provides the normative standard for human nutrition, including vitamins such as A, B1, B2, B6.B12, D, and iodine(Allen, 2012), which all have specific functions to support the human body. Other qualities of breastmilk include the regulation of vascular endothelial growth factor (VEGF)(Loui et al., 2012) (and its versatility in aiding with reducing the burden of retinopathy during prematurity(Lorenz et al., 2009)), prevention of anemia due to containing significant quantities of erythropoietin(Soubasi et al., 1995), metabolism regulation(Newburg et al., 2010), neuron survival, suppressed inflammation, amongst many others(Ballard & Morrow, 2013). Human milk components, such as adiponectin(L. J. Martin et al., 2006), are inversely correlated with infant weight and body mass index. It has been proposed how specific hormones found in breastmilk may contribute to a reduced incidence of overweight and obesity in adulthood.(Woo et al., 2012)

The influence of feeding practices on weight gain has received significant attention, focusing mainly on comparing both sources of nutrition, its components, and the infant's growth trends in association with either feeding practice being followed. As mentioned previously, breastmilk contains hormones. Some (leptin, resistin, and ghrelin) have even been found to play an important role in appetite control, body composition, and regulation of energy conversion. While formula's nutritional value remains the same, breast milk composition changes and evolves to support the nutritional and developmental needs of the infant's rapid growth. While breastmilk is usually twenty calories/oz(SECTION ON BREASTFEEDING et al., 2012b), formula's caloric content may be modified for patients needing higher caloric and protein intakes since concentrations can be adjusted. However, Michealsen and Greer(Michaelsen & Greer, 2014) found that high protein-containing infant formulas were associated with weight gain. It is crucial to consider the evidence research has found to understand better how feeding practices play a role in weight gain and, even presumably, its impact on an individual's health outcomes, even after the first year of life.(Aminoff et al., 2011)

Sleep-wake patterns

It is believed that humans sleep for approximately one-third of their lifetime. Systematic reviews have concluded that short sleep duration is associated with childhood overweight/obesity. The American Academy of Pediatrics (AAP)(*AAP Endorses New Recommendations on Sleep Times | AAP News | American Academy of Pediatrics*, n.d.) has developed evidence-based recommendations around sleep duration guidelines for infants based on age. Infants aged four to twelve months old are recommended to sleep 12-16 hours, followed by 10-13 hours for toddlers aged 1-2, and 10-13 hours for three to five year olds. The AAP has no sleep duration guideline for newborns, but the National Sleep Foundation(Hirshkowitz et al., 2015) recommends 14-17 hours over a 24 hour period. Although the research is limited on sleep patterns, the Brief Infant Sleep Questionnaire-Revised is a standard and validated questionnaire that measures sleep-wake patterns and disturbances in the sleep environment and parental practices around an infant's sleep.(Sadeh, 2004) Data suggest the importance of adequate sleep patterns in infants and how shortened overall sleep duration patterns increase the risk of obesity during toddlerhood(*Zhou Y, Aris IM, Tan SS, et al. Sleep Duration and Growth Outcomes across the First Two Years of Life in the GUSTO Study. Sleep Med. 2015;16(10):1281–1286. 10.1016/j.Sleep.2015.07.006 - Google Search*, n.d.). Analysis of sleep deprivation in adults has been shown to create a hormone imbalance(*The Impact of Sleep and Circadian Disturbance on Hormones and Metabolism*, n.d.) (specifically leptin and ghrelin(Spiegel et al., 2004)-known to be appetite regulators), impairment of proper glucose utilization, and lipid homeostasis(*Short Sleep Duration and Weight Gain: A Systematic Review - Patel - 2008 - Obesity - Wiley Online Library*, n.d.). Although multiple evidence-based studies have addressed the observed link between short duration of sleep and the plausibility of increasing the risk of obesity, limited studies have explored the associations between sleep patterns and rapid weight gain in infants.(*Lavner JA, Stansfield BK, Beach SRH, Brody GH, Birch LL. Sleep SAAF: A Responsive Parenting Intervention to Prevent Excessive Weight Gain and Obesity among - Google Search*, n.d.) There is limited research on sleep deprivation and its association with hormone production in children; data indicates how altered leptin levels(*Hart CN, Carskadon MA, Considine RV, et al. Changes in Children's Sleep Duration on Food Intake, Weight, and Leptin. Pediatrics. 2013; 132:E1473–E1480. - Google Search*, n.d.) in sleep-deprived pre-school-aged children had an increased caloric intake.(*McDonald L, Wardle J, Llewellyn C, et al. Sleep and Nighttime Energy Consumption in Early Childhood: A Population-Based Cohort Study. Pediatr Obes. 2015;10:454–460. - Google Search*, n.d.; *Mullins EN, Miller AL, Cherian SS, et al. Acute Sleep Restriction Increases Dietary Intake in Preschool-Age Children. J Sleep Res. 2017;26:48–54. - Google Search*, n.d.)

The first six months are crucial for understanding how an infant's body adapts to specific factors and structures around sleep duration.(Nishihara et al., 2002) In addition, the first three months are a crucial period for establishing a sleep-wake routine, and sleep-wake timing and circadian rhythmicity along with sleep schedules have been found to influence overall metabolism.(Jarrin et al., 2013) Petrov et al.(Petrov et al., 2021) followed mothers from their first trimester of pregnancy until the infant reached three years of age, analyzing sleep measures and anthropometric measurements when the infant was: one month, six months, and three years. Interestingly, it was found that infants napping more than five times per day had significantly decreased odds of rapid weight gain. Conversely, those sleeping less than five naps/day had greater odds of rapid weight gain.

A question arising from previously discussed research findings is how nutrition impacts overall quality, quantity, and day vs. night sleep during infancy, especially during the first months of life where breastmilk and formula are commonly the only forms of nutrition. A systematic review of 17 articles concluded that substantial gaps exist between investigating the mediating effect of one variable on the other (sleep or diet) and how today's literature on the topic remains inconclusive(Yoong et al., 2016). In a pilot study conducted by Paul et al., parents minimizing feeding for non-hunger responses in order to prolong sleep found that newborns who were predominantly breastfed had more significant nocturnal sleep duration and decreased total daily feeds (including nighttime feeds) at 3,4,8, and 16 weeks compared to mixed or formula-fed infants.(Paul et al., 2011) Contrastingly, three observational studies found that breastfed infants at four months had a more significant number of sleep bouts of shorter duration, with breastfeeding accounting for 39% of the sleep interruptions.(Lampl & Johnson, 2011) Additionally, it was found that increased total daily sleep hours and number of sleep bouts were positively associated with weight gain and accrual of abdominal fat when measuring growth for length. On the other hand, other studies found no correlations between sleep duration and feeding adjustments.(Brown & Harries, 2015; Klingenberg et al., 2013) Studies that have analyzed sleep as the independent variable were interested in finding increased adiposity markers or specific dietary measurements, but none have looked at how diet (specifically breastfeeding, formula, or even solid food introduction) impacts sleep duration. Moreover, the research is remarkably limited for children younger than three years of age.

The Gut Microbiome

The standard/normal adult gut microbiota comprises two significant phyla, Bacteroidetes and Firmicutes.(*Human Gut Microbes Associated with Obesity | Nature*, n.d.) The gut microbiota begins to host microbes the second an individual is born. The microbiome could be describes as an extremely busy city,consisting of trillions of microorganisms (usually referred as microbes) of different species.(Ursell et al., 2012) Some species are more abundant and present than others. Microbes is usually a word correlated to sickness or viruses, but many microbes in the gut play an important role for an individuals health. The human gut microbiome has been of high research interest, and some essential roles it has been found are related to metabolism, nutrition, physiology, and immune defense.(Bull & Plummer, 2014) Each person has a unique microbiome, usually, as mentioned above, the standard gut microbiota is comprised by Bacteroidetes and Firmicutes, however, variations may exist, and some of those can be trailed all the way from infancy.

Microbiome during Childhood

By the age of three, a toddler's intestinal microbiome resembles that of an adult. The gut microbiome undergoes most of its’ development in the first few years of life. Ringel-Kulka et al. compared the intestinal microbiota of children and adults. They found that the abundance of Bifidobacterium, Actinobacteria, Bacilli, and Ruminococcaceae species, were significantly higher in children than in adults, but that the Clostridium cluster XIVa (phylum-like group) was equal between adults and children(*Intestinal*

Microbiota in Healthy U.S. Young Children and Adults—A High Throughput Microarray Analysis | PLOS ONE, n.d.). Such findings suggest how some microbial community members might be established from a younger age, whereas others evolve or change based on outside factors. Generally, microbial diversity has been observed to be lower in children aged 3-4 and 5 years of age when compared to adults.(De Filippo et al., 2010) A study in which 28 children's microbiota at five years of age was analyzed found the same taxa abundances as the previous study conducted by Ringa-Kulka et al. (De Filippo et al., 2010)

Microbiome Development During the First Months of Life

The microbiome begins to develop the second an individual is born. The gut microbiota partakes in different functions and is shaped by early life events such as mode of delivery- cesarean vs. vaginal, early antibiotic exposure, feeding practices, and sleep-wake patterns. Both gut and vaginal microbes are the primary sources of the initial transfer of microbes to a newborn.(Nuriel-Ohayon et al., 2016) The vaginal(“Structure, Function and Diversity of the Healthy Human Microbiome,” 2012) and gut microbiome fluctuate throughout pregnancy, creating unique environments for a newborn to be exposed to if born via vaginal birth. The mother's gut microbiome has also changed even before delivery. For example, a study conducted by Koren et al. observed dramatic changes from the first trimester to the third trimester, -observing changes in diversity and an overall increase in Proteobacteria and Actinobacteria in relation to gestational age.(Koren et al., 2012) During the first trimester, it has been observed that the gut microbial composition is similar to one of a non-pregnant woman, with a predominance of Firmicutes, mainly clostridial species and lower abundance of Bacteroidetes.(Walters et al., 2014) By the third trimester, the maternal gut declines in butyrate-producing bacteria and increases in Proteobacteria, Actinobacteria, Bifidobacteria, and lactic-acid producing bacteria(Koren et al., 2012). Early microbial seeding, in other words, early exposure to microbes, may play potential long-term health benefits for the newborn. A sample of 396 healthy women revealed that lactobacilli are primary vaginal bacteria and their dominance persists into pregnancy.(Ravel et al., 2011)

The vaginal microbiome is characterized by low diversity and high stability.(Palmer et al., 2007) Lactobacillus creates a protecting barrier against ascending infections,(Aagaard et al., 2012) and its' dominance may represent an adaptation favoring colonization of offspring with beneficial microbes during delivery.(Romero et al., 2014) Community structure differences and characteristics have been observed when comparing vaginal vs. cesarean delivery. Vaginally delivered infants are introduced to *Bacteroidetes, Lactobacillus, Bifidobacterium*, and *Parabacteroides*.(Bäckhed et al., 2015, 2015; Dominguez-Bello et al., 2010) A higher abundance of Bifidobacterium amongst vaginal-delivered newborns remains consistent in the literature. (Bäckhed et al., 2015)These microbes are known to promote gut health and defend against pathogen invasion. In contrast, children born via cesarean show a delay in colonization by such bacteria(Reyman et al., 2019) which may contribute to the greater likelihood of immune-related disorders(*Planned Repeat Cesarean Section at Term and Adverse Childhood Health Outcomes: A Record-Linkage Study - PubMed*, n.d.), including asthma/allergies(*Cesarean Section and Disease Associated with Immune Function - ScienceDirect*, n.d.), inflammatory bowel disease,(*Cesarean Section and Disease*

Associated with Immune Function - ScienceDirect, n.d.) and rapid weight gain.(H. -t Li et al., 2013)

Feeding practice influences on the infant gut microbiome

Feeding practices are essential for an infant's growth. As discussed above, both breastmilk and formula contain different nutritional characteristics that affect overall health. Besides specific nutrients, sugars, or proteins, both feeding practices play a role in the development and maintenance of the gut microbiome's community structure. Human breastmilk has been found to have a wide array of benefits to an infant not only during their newborn stage but across toddlerhood. It is evident that breastmilk contains unique microbes that cannot be manufactured in a laboratory setting to seed and shape the gut microbiome from birth adequately.(Pannaraj et al., 2017) Some components, such as human milk oligosaccharides, have been found to serve as a prebiotic to promote the growth of *Bifidobacterium*(Sela et al., 2008). Some strains within *Bifidobacterium*, such as *B.infantis*, can transport human milk oligosaccharides into the cytoplasm to adequately digest them.(Ruiz-Moyano et al., 2013; Sela et al., 2011) Microbes such as *B.infantis* have been positively associated with immune protection by enhancing adherence to beneficial intestinal cells and inhibiting undesirable microbial strains(Bernet et al., 1993), producing anti-inflammatory cytokines,(Chichlowski et al., 2012; O'Hara et al., 2006) and inhibiting the translocation of gram-negative bacterial toxins by increasing acetate production, a short-chain fatty acid derived from carbohydrate fermentation.(Fukuda et al., 2011)

Different microbes have been found in breastmilk samples, suggesting that composition is highly individualized and dependent on variables such as geographic location(Kumar et al., 2016) and genetics.(Pärnänen et al., 2018) For example, Hunt et al(*Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk | PLOS ONE*, n.d.). sequenced microbial DNA in milk from sixteen women at different timepoints over four months. Only nine microbial genera were identified across all lactating women's breastmilk, *Staphylococcus, Streptococcus, Serratia, Pseudomonas, Corynebacterium, Ralstonia, Propionibacterium, Sphingomonas, and Brazyhizobiaceae.* Other studies analyzing different groups of women documented different microbial findings, such as *Baltic, Brevundimonas, Flavobacterium, Rothuia, Burkholderia, Elizabethkingia, Variovorax, Enhydrobacter*.(Murphy et al., 2017)

Dissimilarities in the milk's microbial composition may be attributed to various reasons, including diet, genetics, demographics, or environment. A study comparing cesarean vs. vaginal birth infants found a higher overall bacterial concentration in breastmilk of *Streptococcus* and lower *Bifidobacterium* concertation for cesarean births.(Khodayar-Pardo et al., 2014) Antibiotic use influences the gut microbial structure, and even intrapartum antibiotic use during an infant's delivery changes maternal milk microbial composition.(Hermansson et al., 2019) High values of maternal body mass index and pregnancy weight gain have also been associated with lower bacterial diversity in colostrum.(Cabrera-Rubio et al., 2012) Even though differences in breastmilk microbial compositions are evident, several studies have observed that there is a mother to infant vertical transfer of bacterial species(Lyons et al., 2020), which means that the microbes found in lactating mothers will eventually be found in an infants' gut microbiome, such as *Bifidobacterium breve* and *Lactobacillus Plantarum*. (Murphy et al., 2017)

Introduction of Solid Foods and the Gut Microbiome

Besides breastfeeding and formula-feeding, an emerging topic that has has started to receive scientific attention is the timing and quality of solid foods introduction in the first year of life and how it affects the gut microbiome. The introduction of solid foods into an infant's diet causes a dramatic shift in the microbiome due to allowing the entry of new microbes found in food that are usually not found in breastmilk or formula. The composition of gut microbes in the infant is believed to be directly related to nutritional intake but remains understudied regarding solid foods. However, it has been observed how microbial richness (via observed species) and diversity (via Shannon Index) increased over time with dietary diversity introduced into an infant's diet.(Homann et al., 2021) One phyla that has been observed to increase is *Bacteroidetes,* which is one microbial group commonly found in adults. Another study observed an increase in *Ruminococcus* and a decrease in *Escherichia* richness in the introductory period of solid foods; however, no correlations were created to the kinds of food ingested.(Vallès et al., 2014)(Vallès et al., 2014) Homann et al. analyzed the impact of solid food introduction on gut microbiome shift in twenty-four babies in intercontinental cohorts in Canada (S. Kim et al., 2017)and the Netherlands(de Korte-de Boer et al., 2015). Food diaries were collected daily, besides a stool sample collected prior to the solid's introduction, and multiple were collected after. The day-to-say sampling, via DNA extraction and V3 16S rRNA gene amplification from diaper collection in both cohorts, allowed to understand and open the opportunity to create correlations of the day-to-day changes and exposure via having an overview of the average nutritional intake.

The age and timing of solids foods are additional factors to consider. In the Netherlands'-based cohort (Lucki Gut), the average age was 5.2 months, compared to the Canadian cohort (Baby, Food, & MI) which was 5.5 months. the Canadian infants, fiber intake was positively associated with increased microbial richness. Overall, the introduction of solids seemed to have a strong impact on an infant's gut microbial community and requires further study.

Sleep patterns

It has been evidenced how the gut has a connection with the brain, called the gut-brain axis. This connection appears to be bidirectional, affecting multiple systems in the human body, including the endocrine, immune, and nervous systems. Disruptions in the gut microbiome have been associated with psychological disorders, including anxiety and depression. More recent research has explored how sleep behaviors also impact the gut microbiome and vice versa. A diverse microbiome has been associated with adequate hours slept in a 24-hour period with minimal sleep disruptions.(Smith et al., 2019) Specific microbes have also been linked to sleep outcomes. For example, Smith et al. found that the richness of *Bacteroides and Firmicutes* was positively correlated with sleep efficiency and its' distribution may be altered from partial sleep deprivation.(Benedict et al., 2016; Poroyko et al., 2016) Reduction in *actinobacteria* correlated with increased sleep disruption(Poroyko et al., 2016), while increased richness had a negative correlation with the total number of awakenings.(Smith et al., 2019) Besides sleep practices influencing overall composition, inflammation, circadian misalignment, and sleep loss are associated with gut dysbiosis and may cause microbial imbalances in alpha and beta diversity.(Y. Li et al., 2018)

Overall, sleep quality in adults is influenced by many contributing factors and could cause constant changes in the gut microbiome; however, changes in the gut microbiome phyla in younger populations in relation to their sleep-wake patterns remain understudied.

The use of breastmilk, antibiotic exposure, and type of birth have been shown to shape the gut microbiome. Currently, the sleep-gut connection during infancy remains understudied, but emerging data has shown prominent findings on how specific sleep behaviors may profoundly affect the gut microbiome from a young age. With sleep regulation maturing during the first year of life by establishing a 24-hour rhythm and day vs. night sleep schedules,(Schoch et al., 2020; *Sleep Duration From Infancy to Adolescence: Reference Values and Generational Trends | Pediatrics | American Academy of Pediatrics*, n.d.) the gut has been proposed to play a role in such patterns primarily due to observation of how early microbe colonization is linked to brain signaling(Heijtz et al., 2011). A recently published study in Europe conducted by Schoch et al. focused on capturing all aspects of infant sleep by creating five different sleep composites: sleep activity, sleep timing, sleep at night, sleep during the day, and sleep variability. Sleep habits were measured at three, six, and twelve months during continuous days with ankle actigraphy and a twenty-four-hour diary protocol(Schoch et al., 2022)l; to have a more accurate reporting of the sleep habits. Stool DNA extraction and 16s rRNA-gene amplicon sequencing demonstrated a previously supported gut microbial abundance for an infant: the presence of Bifidobacterium and Bacteroides. Infants with less predominant daytime sleep had a higher alpha diversity + nighttime sleep fragmentation and variability were linked with bacterial maturity and enterotype. In addition, this test demonstrated how the sleep-brain-gut is relevant in development, therefore making infancy a prime time for how sleep habits and bacterial markers predict behavioral-development outcomes later in life. Schoch et al. findings played a fundamental role to support and validate the emerging concept of sleep-brain-gut axis; demonstrating how the gut is heavily intertwined and more research should be done on this. Especially, since both sleep and gut microbiota variables are easily and non-invasively modified. More literature and finding supporting this could support how adequate sleep and a healthy gut during infancy can be part of a bigger health outcome and determinizing factor across the lifespan.

Another question that arises is how nutrition works in conjunction with sleep. Since it has been observed how sleep duration and regularity may have an apparent effect dietary intake. Such combinations have yet to be explored. An infant's gut constantly changes and develops, especially when more environmental factors come into play. As many diseases are rooted in childhood, it is imperative to continue researching how modifiable practices may be seeding the overall composition and diversity of the gut microbiome and how this relates to downstream health outcomes.

CHAPTER 3

METHODS

Participants and Study Design

This study is a secondary analysis of data from a randomized longitudinal intervention study in which the efficacy of a home-based education program was assessed for preventing the onset of childhood obesity.(Whisner et al., 2019) A convenience sample of 40 mother and infant dyads were recruited to participate in an additional collection of fecal samples to evaluate associations between lifestyle/behavioral factors in infancy and gut microbiome composition in toddlerhood.

The female subjects that were included in the study self-identified as Mexican American, were between the ages of 18-40 years, had a pre-pregnancy BMI of $\geq$25 kg/m^2 (which is an indication of overweight), and with no diagnosed medical conditions that could interfere with the growth and health of the infant during gestation. After birth, infants were enrolled in the study if they were born at 38 weeks of gestation or later, had a birth weight $\geq$2500 grams, and did not have any genetic or medical conditions that could interfere with growth outcomes.

The subjects were recruited from Special Supplemental Nutrition Assistance Program for Women, Infants, and Children (WIC) clinics. All women in the study continued to receive any support they were seeking from this institution, such as: breastfeeding assistance, nutrition counseling, and vouchers for formula or any additional nutritional information they requested. The mothers were randomized at 36 weeks of gestation to either (1) a parent education program in which trained Promotoras or community health workers counseled parents on different topics such as: feeding practices and infant growth or, (2) a control group in which they received measurement visits without intervention. The study was approved by the Arizona State University Institutional Review Board (IRB#1207008084).

In total, there were ten study intervention visits that occurred prenatally and postpartum across the first 24 months of the infant's life. The educational content presented to the mothers included education on growth monitoring, feeding support, parenting styles, and adequate sleep. At eight times, blinded, bilingual research assistants (RA) visited participant homes to take growth measurements. The first of these visits started during the first week of life and continued until 36 months of age. Infant growth was measured by obtaining infants' supine weight on a portable electronic scale (Seca Scale models 869-1321004 and 19-17-05-224) to the nearest 0.1 kg and length measurements on a portable recumbent length board estimated to the nearest 0.1 cm. RWG was defined as an increase in weight-for-age Z-score of greater than 0.67 at six months of age.(Ong & Loos, 2006)

Sleep-wake pattern analysis

The primary independent variable in this secondary analysis is sleep patterns. To compile subjective data about infant sleep patterns, the Brief Infant Sleep Patterns questionnaire was administered to the mothers at 1, 6 and 12 months. The questionnaire contained a series of questions that assessed sleep quality and quantity. Some of the questions included: total sleep throughout the day, where/when the infant slept, total amount of uninterrupted sleep, and overall sleep quality of the infant. To categorize adequate total 24-hour sleep time data for the infant, recommendations from the National Sleep Foundation, American Academy of Pediatrics, and American Academy of Sleep

Medicine were used.(Hirshkowitz et al., 2015) The National Sleep Foundation recommendations are as follows: ages 0-3 months: 14-17 hours , ages 4-11 months: 12-15 hours , 12-15 hours for infants(*How Much Sleep Do Babies and Kids Need?*, 2020) aged 4-11 months and ages 1-2 years: 11-14 hours; whereas, the American Academy of Pediatrics and American Academy of Sleep Medicine(Paruthi et al., 2016) sleep recommendations are: ages 4-12 months: 12-16 hours; ages 1-2years: 11-14 hours. Since there is an overlap of recommended guidelines, the total sleep parameters used for this analysis were categorized as follows: 14-17 hours for infant at one month of age, 12-16 hours for infant at six and 12 months of age.

Feeding modes

A breastfeeding intensity scale was administered by telephone by the RA at monthly intervals until the mother weaned the infant from the breast. In addition to the breastfeeding intensity scale, infant food intake was recorded at one month, six months and 12 months of age. The breastfeeding intensity scale assessed breastfeeding status, frequency, duration and intensity, quantity of any formula consumed, and the age when the infant was introduced to solid foods. Because feeding mode (breastfed vs formula-fed) has been associated with sleep-wake patterns, this information will be included in models to explore potential interactions with infant sleep and gut microbiota composition.

Fecal Collection

Infant stool samples were collected via sterile swab (Copan FLOQSwabs, Murrieta, CA) by the parent and/or guardian at 36 months of age. The parent and/or guardian was instructed to collect the child’s stool sample by swabbing a dirty diaper or when wiping the child’s buttocks and placing the swab in a sterile tube. Afterwards, the swab was placed in a sterile tube. The sterile tube was placed in a biohazard bag and refrigerated at 4°C until research staff collected the sample. After collection, the research staff overnight shipped samples to ASU. Upon arrival at the ASU Clinical Research Unit, samples were stored at -80°C to preserve microbial communities for future processing. The sample swab was then used to extract microbial DNA for sequencing.

DNA Extraction and Sequence Data Analysis

The swab from the child stool collection was removed from the sterile tube after defrosting on ice. The swab tip was carefully removed with sterile scissors and placed into a PowerBead® tube, and vortexed until complete homogenization of the sample was obtained. The sample was then transferred to a collection tube and the PowerBead® tube with the swab was discarded. Microbial DNA was extracted using the PowerSoil® DNA isolation kit following the manufacturer's protocol (MoBio Laboratories Ltd., Carlsbad, CA) and an additional cleaning step (protocol provided by MoBio) to remove additional inhibitors common in feces that might influence downstream sequencing. Amplification of the 16S rRNA gene sequence was completed in triplicate PCRs using 96-well plates by laboratory staff at the Biodesign Institute at ASU. The V4 region of the 16S rRNA gene was amplified by utilizing forward 515F primers and 806R reverse primers containing Illumina adapter sequences. These primers were applied as recommended by the Earth Microbiome Project.(Caporaso et al., 2011),(Caporaso et al., 2012) Previously outlined protocols for PCR amplification, amplicon cleaning, and quantification were performed.(Caporaso et al., 2012) Pooled individual samples of equimolar ratios of amplicons were sequenced on the Illumina platform (Illumina MiSeq instrument, Illumina, Inc., San Diego, CA) at ASU's DNASU Genomics Core Facility. Raw Illumina microbial data were cleaned using Quantitative Insights Into Microbial Ecology (QIIME) software, Version 2020.2, as previously described.(Callahan et al., 2016) Samples were quality checked and denoised to correct sequencing errors using DADA2; filtering, dereplication, and merging of paired-end reads were also performed using DADA2 (Callahan et al., 2016). Taxonomic assignment of amplicon sequence variants (ASVs) were determined utilizing the closed reference Silva 138 99% OTUs from 515F/806R region of sequences at 99% similarity.(*Optimizing Taxonomic Classification of Marker-Gene Amplicon Sequences with QIIME 2's Q2-Feature-Classifier Plugin | Microbiome | Full Text*, n.d.; Quast et al., 2013) Sequence alignment was performed using MAFFT (Katoh & Standley, 2013). Fasttree (default) was used to produce a phylogenetic tree for diversity analyses (Price et al., 2010).

Statistical analysis

All statistical analyses were completed using the Statistical Package for the Social Sciences 25 (SPSS, Inc., an IBM Company, Chicago, Illinois, USA) and QIIME 2020.2 statistical and bioinformatics software packages. Data were expressed as mean±SD or median (interquartile range) for demographic, anthropometric, and microbiota frequencies/proportions based on the normality of the data.

Independent variables related to infant feeding and sleep behaviors will be analyzed using categories. The category of "breastfed" on one, six and twelve months will include any breastmilk received as described above (including formula feeds) be placed into variable, and infants receiving only formula will be placed under "formula-fed." Although there is more support for having an established routine past the first month of life where the newborn is still adapting to its' new environmental surroundings for adequate total 24-hour sleep recommendations/ranges, this age period will still be taken into consideration and assessed since more mothers provided breastmilk to infants compared to the rest of the age timepoints; as well because the Brief Infant Sleep Patterns questionnaire was also administered during that age. Total sleep duration at one, six, and twelve months (meets/ does not meet recommended total twenty-four-hour sleep ranges as follows: one month: 14-17 hours, 6 months and 12 months: 12-16 hours. These variables will be assessed at one, six months, and 12 months of age. Total 24-hour sleep (including day + night sleep duration) along with feeding methods will be merged into one variable with four different categories (adequate sleep + breastfed; adequate sleep + formula fed; inadequate sleep + breastfed; and inadequate sleep + formula fed).

Group comparisons will be performed for each independent variable during infancy (described above) with gut microbiome alpha and beta diversity at 3 years of age as the dependent variable. Phylogenetic diversity metric analyses will be performed in QIIME2 to determine alpha (within-sample) diversity via Shannon's Index, a measure of ASV richness and evenness. In addition, observed OTUs, and Faith's PD will be performed for additional alpha diversity analysis. Median alpha-diversity values were visually reported using distribution comparison plots (box-and-whisker plots), and group differences evaluated by Kruskal-Wallis tests (Kruskal & Wallis, 1952). Principal coordinate analysis (PCoA) and EMPeror were used to visually assess group differences in beta (between-sample) diversity using Bray-Curtis dissimilarity, Jaccard distance, weighted and unweighted unique fraction (UniFrac) metrics on the 99% ASV composition and abundance matrix. Statistical group differences will be tested with PERMANOVA after 999 permutations and pairwise comparisons.

All metrics used in the beta diversity analyses accounted for the presence and absence of microbial taxa, though only the Bray-Curtis and Weighted Unifrac metrics accounted for taxa abundance. Phylogenetic ß-diversity metrics (Unweighted and Weighted Unifrac measures) were used to evaluate evolutionary relationships between taxa, whereas Jaccard and Bray-Curtis measures assumed equal relationships between present taxa.

Statistical Analysis by Hypothesis

H1: Alpha diversity in sleep wake patterns at one, sixth and twelve months of age will be assessed independently, as well as in combination with feeding practices (any breastmilk or exclusive formula) via Shannon's Diversity Index to meet both hypothesized aims. This quantitative measure will assess community richness between groups and analyze for any statistical significance with a p value of .05.

H2: Beta diversity in in sleep wake patterns at one, sixth and twelve months of age will be assessed independently, as well as in combination with feeding practices (any breastmilk or exclusive formula) via Jaccard distance and Bray-Curtis distance. Both qualitative (Jaccard) and quantitative (Bray-Curtis) measures of community's dissimilarity will assess statistical significance with a p value of .05 using pairwise permanova statistics between groups.

CHAPTER 4

RESULTS

Participant Characteristics

A total of 40 infants (52.5% male) provided a fecal sample at 36 months of age. The mean household size was 4.9±.1.9 family members with an average of 2.2±1.5 children per household. The majority (55%, n=22) of households had an average annual income between $20,000 to $29,999. The majority (42.5%, n=17) of infants had mothers with a middle school education who were primarily born in Mexico (70%, n=28). Additionally, the mean maternal pre-pregnancy BMI was 34.1±5.7 kg/m^2 with an average weight gain of 26.8±11.4 pounds during pregnancy. Analysis for sleep and feeding practices at one month of age were calculated with a total sample size of n=39, due to missing data for feeding type and total sleep during 24 hours from one infant at this time point, however, for six and twelve months, data were available for the total sample population (n=40).

Infant Feeding Practices

Most infants (77%, n=30) received some or partial breastfeeding at one month of age, and 40% (n=12) were exclusively breast-fed. Rates of infants receiving any breastmilk decreased over the three time periods; 65% (n=26) and 55% (n=22) received some or partial breastfeeding at six and twelve months of age, respectively. Of the initial twelve infants who were exclusively breastfed at one month, five infants (45%) remained exclusively breastfed at sixth months, which further decreased to three infants (25%) at twelve months. Breastmilk and formula were the only reported nutrition that were provided for infants during one and six months of age;

however, cow's milk was then provided to some infants at twelve months of age (41%, n=16). Although solid introduction was not assessed as a variable for this specific analysis, the parent study did collect timing and type of solid introduction. The majority of infants (93%, n=37) were introduced to solids prior to or at six months of age with a mean age of 4.8±1.0 months.

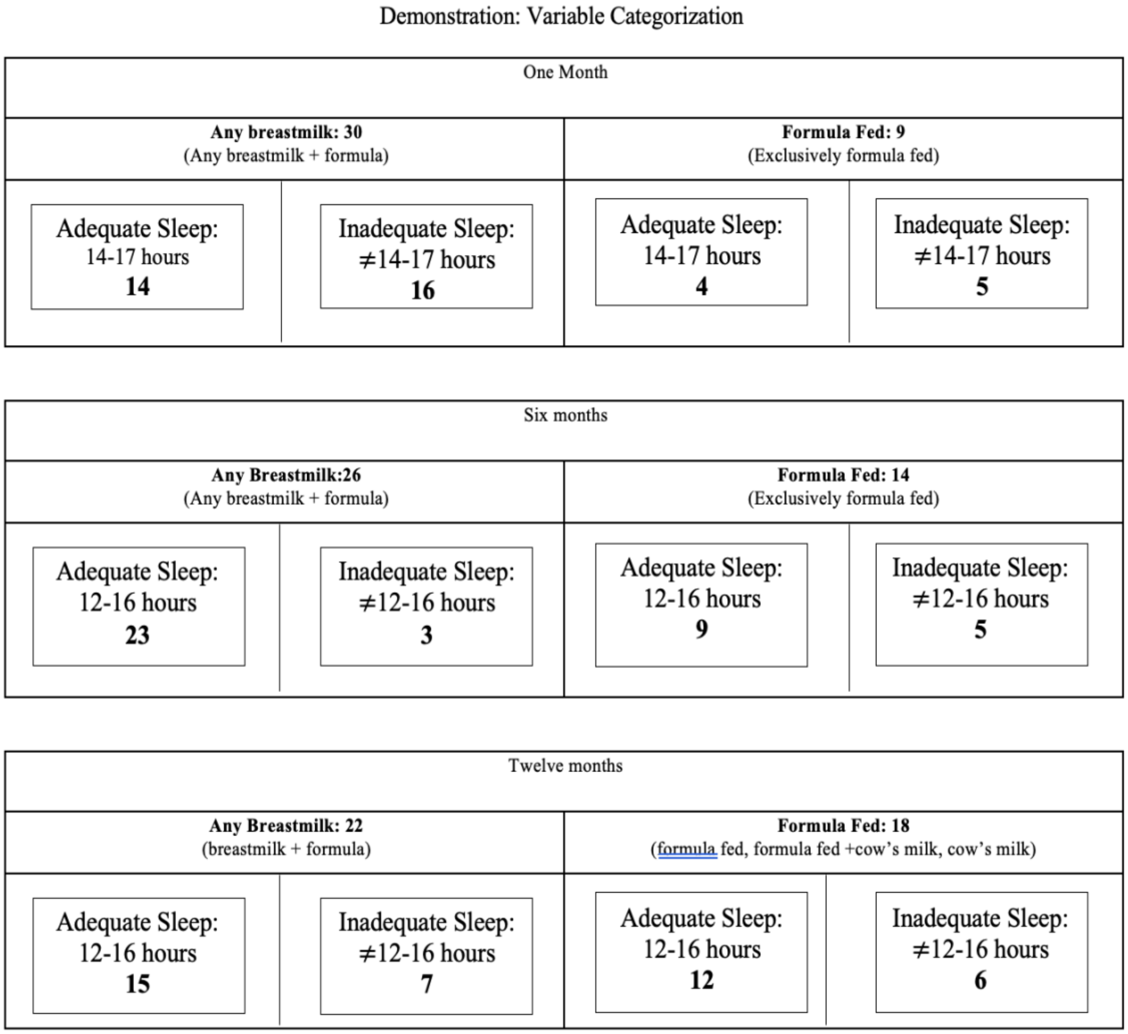
Infant Sleeping Practices

Sleep data were obtained from the administration of the Brief Infant Sleep Questionnaire during the three age periods (one, six and twelve months). The questionnaire assessed the infant's bedtime routine, total amount of naps, total hours slept at night and throughout the day, level of difficulty of putting infant to sleep, and even the sleep quality of an infant. Total hours slept in a 24-hour period were assessed and categorized as adequate vs inadequate sleep, with a further breakdown and variable categorization analyzing feeding practices and sleep adequacy/inadequacy together which can be found in **Table 1**. At one month, the mean total time slept was 16.9±3.3 hours. Of the 30 infants receiving any breastmilk, 47% (n=14) were categorized as receiving adequate sleep (categorization of total sleep between 14-17 hours) compared to 53% (n=16) receiving inadequate sleep (less than 12 hours or more than 17 hours of sleep in a 24-hour period). Formula fed infants at one month had similar sleep patterns, with 44% (n=4) receiving adequate and 56% (n=5) receiving inadequate sleep. At six and twelve months of age, it was observed that more infants received adequate sleep in both the breastfed and formula fed groups (adequate sleep being 12-16 hours total and inadequate equaling to less than 12 or more than 16 hours of total sleep). Twenty-three (88%) of the infants receiving any breastmilk at six months of age had adequate sleep, and fifteen

(68%) at twelve months, respectively. Formula fed infants also received more adequate sleep at both six (64%, n=9) and twelve (67%, n=12) months. Additionally, there was a trending decrease in the average hours slept as the infants became older, equaling 13.6±2.1 hours at six months, and 12.5±1.8 hours at twelve months.

Table 1

Variable Categorization: Feeding Practices and Sleep Adequacy/Inadequacy



Demonstration: Variable Categorization

One Month			
Any breastmilk: 30 (Any breastmilk + formula)		**Formula Fed: 9** (Exclusively formula fed)	
Adequate Sleep: 14-17 hours **14**	Inadequate Sleep: ≠14-17 hours **16**	Adequate Sleep: 14-17 hours **4**	Inadequate Sleep: ≠14-17 hours **5**

Six months			
Any Breastmilk:26 (Any breastmilk + formula)		**Formula Fed: 14** (Exclusively formula fed)	
Adequate Sleep: 12-16 hours **23**	Inadequate Sleep: ≠12-16 hours **3**	Adequate Sleep: 12-16 hours **9**	Inadequate Sleep: ≠12-16 hours **5**

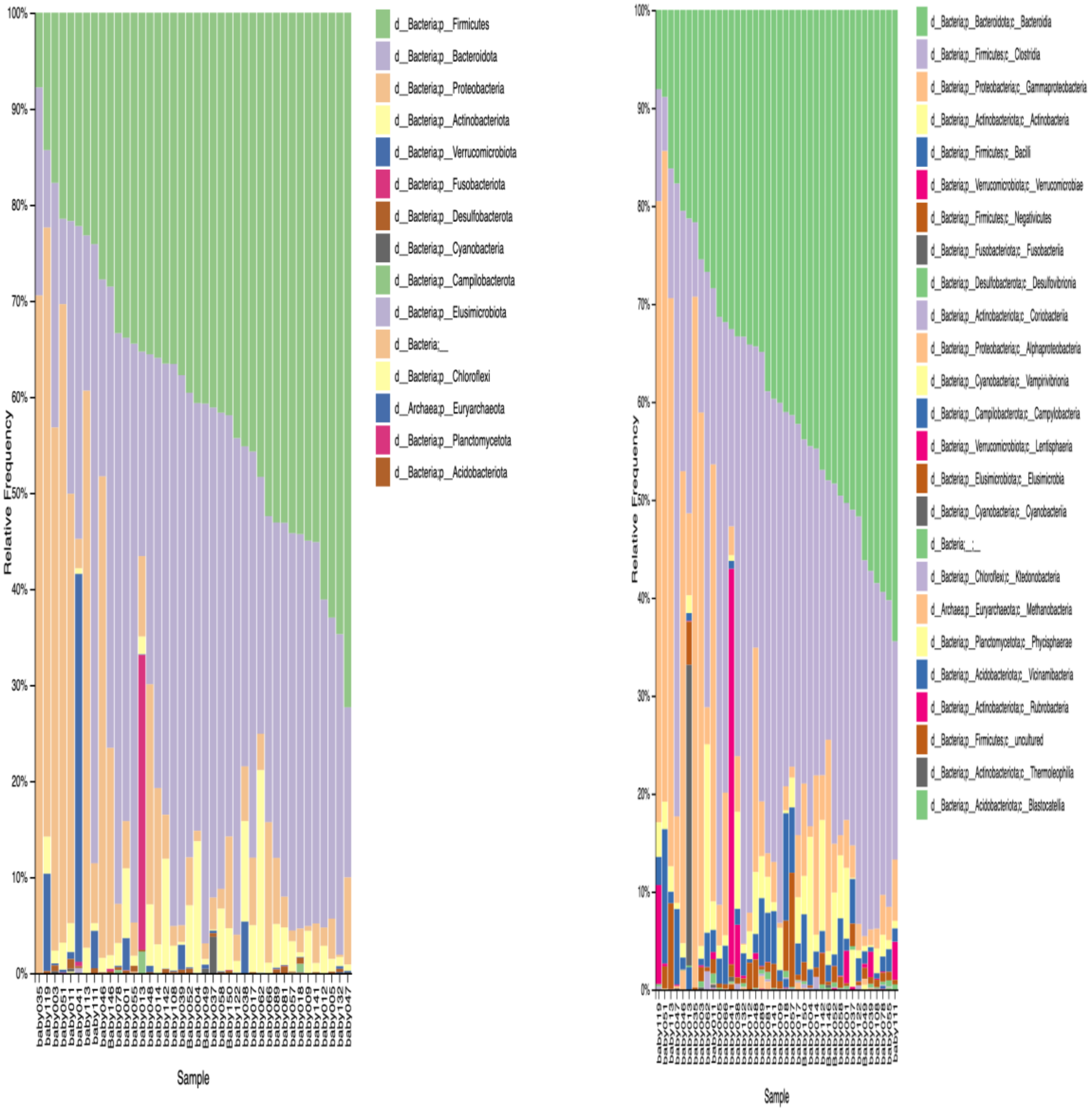
Twelve months			
Any Breastmilk: 22 (breastmilk + formula)		**Formula Fed: 18** (formula fed, formula fed +cow's milk, cow's milk)	
Adequate Sleep: 12-16 hours **15**	Inadequate Sleep: ≠12-16 hours **7**	Adequate Sleep: 12-16 hours **12**	Inadequate Sleep: ≠12-16 hours **6**

Gut Microbiome

All 40 participants' fecal samples were collected at 36 months of age. Amplicon high-throughput sequencing resulted in an average of 29,865 16s rRNA gene amplicon reads per sample. Before completing the data analysis, the data were normalized for varying sequence depths across samples via rarefaction. DADA2 was used for sequence trimming and performed at 7 and 205 bases for forward reads and 6 and 196 bases for reverse reads; this was based on the visual evaluation of the sequence quality data with achievement of median quality scores above 35. Rarefaction curves based on alpha diversity suggested that the adequate sampling depth was at 4027 sequences minimum and 21016 sequences maximum. Various alpha measures including observed OTU's at 1, 6, and 12 months comparing sleep and feeding modality groups and Shannon Diversity comparing sleep adequacy were developed to decide on a rarefaction depth to create equal sampling across all samples. The median frequency per sample was 17,428 which did not significantly differ by sleep or feeding practices. Taxonomic bar plots were also obtained to show differences in taxa levels (phylum and order) at 36 months. **(Figure 1**)

To measure alpha and beta diversity, fecal samples were analyzed by sleep adequacy (adequate vs inadequate sleep variables) and sleep adequacy combined with feeding practices at one, six, and twelve months. There were in total two groups for each diversity analysis when comparing adequate and inadequate sleep and a total of four group comparisons after combining sleep adequacy/inadequacy and formula fed/breastfed.

Figure 1: Taxonomic Bar Plots: Phylum & Order



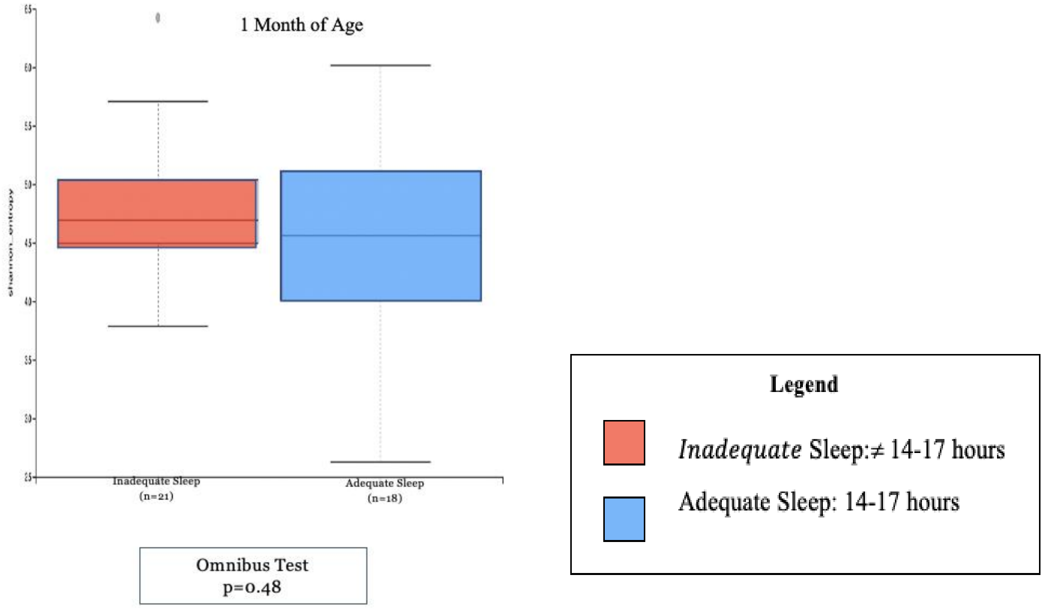
<u>**One Month of Age:**</u>

Alpha and Beta Diversity Metrics for Sleep Adequacy & Feeding Modalities

Alpha Diversity Metrics: Shannon’s Index

Infants who had inadequate sleep (≠14-17 hours) at one month had slightly higher alpha diversity (Shannon’s Index ,**Figure 2)** at 36 months of age; however no significant differences between groups with differing sleep practices were found (p=0.48, H=0.49).

Figure 2: Shannon Diversity Measuring Sleep Adequacy at One Month



Infants with adequate sleep patterns but differing feeding practices (any breastmilk/formula) at one month had no differences (p=0.77, H=1.78; **Figure 3 & Table 2)** in within-sample gut microbiome richness (Shannon’s Diversity) at 36 months of age.

Figure 3: Shannon Diversity Kruskal Wallis Pairwise Difference for Sleep + Feeding Modality at One Month of Age

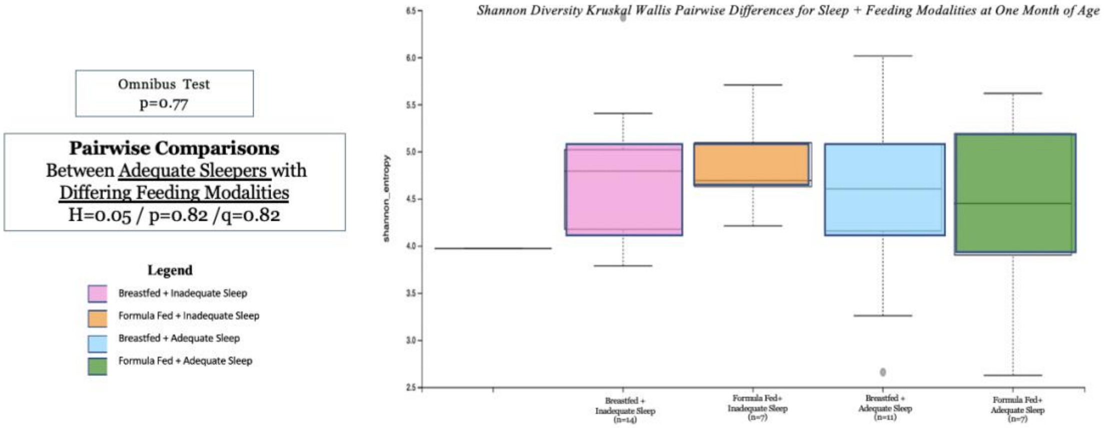
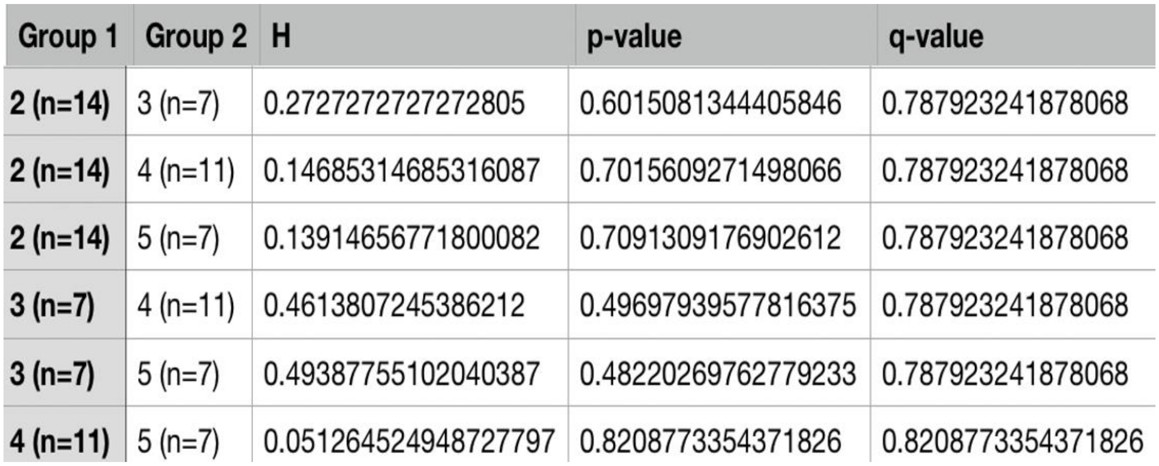


Table 2: Shannon Diversity Kruskal Wallis Pairwise Comparison Test Results for Sleep + Feeding Modality at One Month of Age and Gut Microbiome at 36 Months of Age



Group 1	Group 2	H	p-value	q-value
2 (n=14)	3 (n=7)	0.2727272727272805	0.6015081344405846	0.787923241878068
2 (n=14)	4 (n=11)	0.14685314685316087	0.7015609271498066	0.787923241878068
2 (n=14)	5 (n=7)	0.13914656771800082	0.7091309176902612	0.787923241878068
3 (n=7)	4 (n=11)	0.4613807245386212	0.49697939577816375	0.787923241878068
3 (n=7)	5 (n=7)	0.49387755102040387	0.48220269762779233	0.787923241878068
4 (n=11)	5 (n=7)	0.051264524948727797	0.8208773354371826	0.8208773354371826

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

Beta Diversity Metrics: Bray-Curtis, Jaccard Distance, and Unweighted Unifrac

Between-sample (beta) diversity metrics included Jaccard (for qualitative, non-phylogenetic analysis) and Bray-Curtis (for quantitative, non-phylogenetic) distances. There were no significant differences (p=0.36) observed for Bray Curtis metrics at 36 months of age for either sleep adequacy alone (**Figure 4)** or for sleep adequacy plus feeding modalities (p=0.57) at one month, as seen on **Table 3.**

Figure 4: Bray Curtis Kruskal-Wallis Pairwise Differences for Sleep Adequacy at One Month

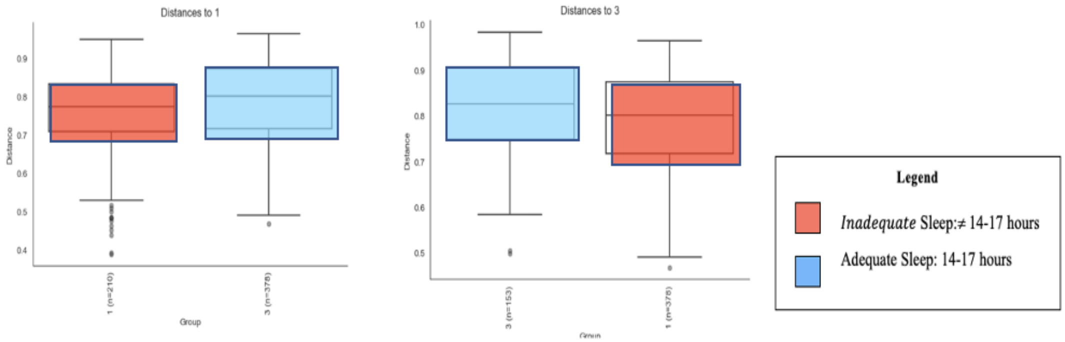


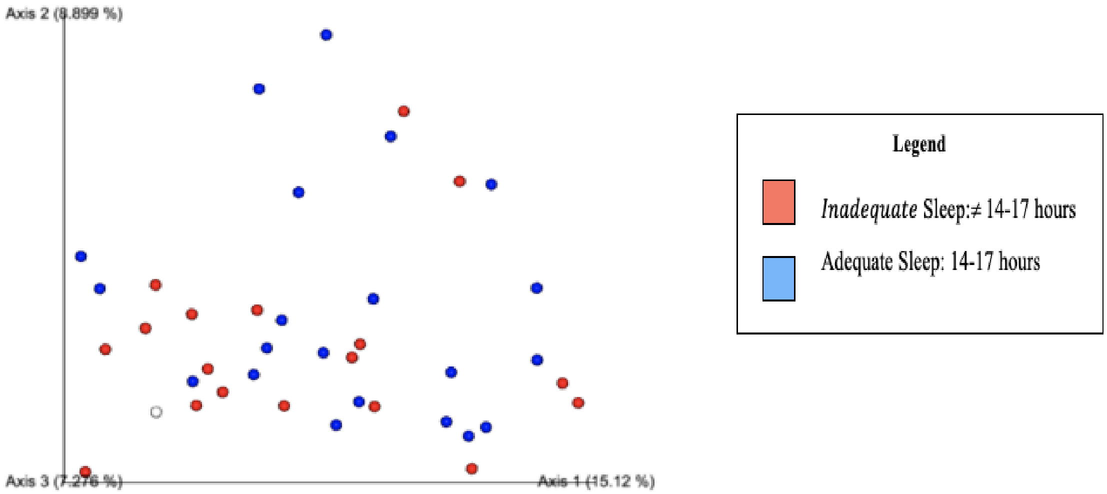
Table 3: Bray Curtis Pairwise Comparisons for Sleep + Feeding Modality at One Month of Age

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
2	3	21	999	0.859856822567339	0.626	0.7825
2	4	25	999	1.0694000186839352	0.328	0.7825
2	5	21	999	1.2090460685401827	0.186	0.7825
3	4	18	999	0.8288480928225175	0.75	0.8333333333333333
3	5	14	999	0.7060864604250202	0.884	0.884
4	5	18	999	0.9587221227990583	0.526	0.7825

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

An emerging trend was observed (p=0.09) in overall microbial community structure in toddlerhood when evaluated using the Jaccard metric in relation to sleep adequacy at one month. This trend disappeared after analyzing Jaccard Distances in relation to sleep and feeding modalities (p=0.17). Additional beta-diversity metrics had a trending differences by one-month sleep groups (Unweighted Unifrac, p=0.06, **Figure 5**) suggesting that the overall microbial community structure differed in toddlerhood as a result of total sleep achieved at one month of age.

Figure 5: Emperor Unweighted Unifrac for Sleep Distribution at One Month



<u>Six Months of Age:</u>

Alpha and Beta Diversity Metrics for Sleep Adequacy & Feeding Modalities

Alpha Diversity Metrics: Shannon’s Index

Inadequate sleep at 6 months (Shannon’s Diversity Index, p=.052; **Figure 6**) also had a trending association with gut microbiome richness in toddlerhood. When community richness was evaluated with sleep adequacy/inadequacy and feeding modalities together, a trending significant value of p=0.06, H=7.09 (**Figure 7)** was observed. Per **Table 4**, formula feeding at 6 months of age seemed to result in a greater difference in microbial richness by sleep adequacy categories (q=.01,H=8.6).

Figure 6: Shannon Diversity Index for Sleep Adequacy at 6 Months of Age

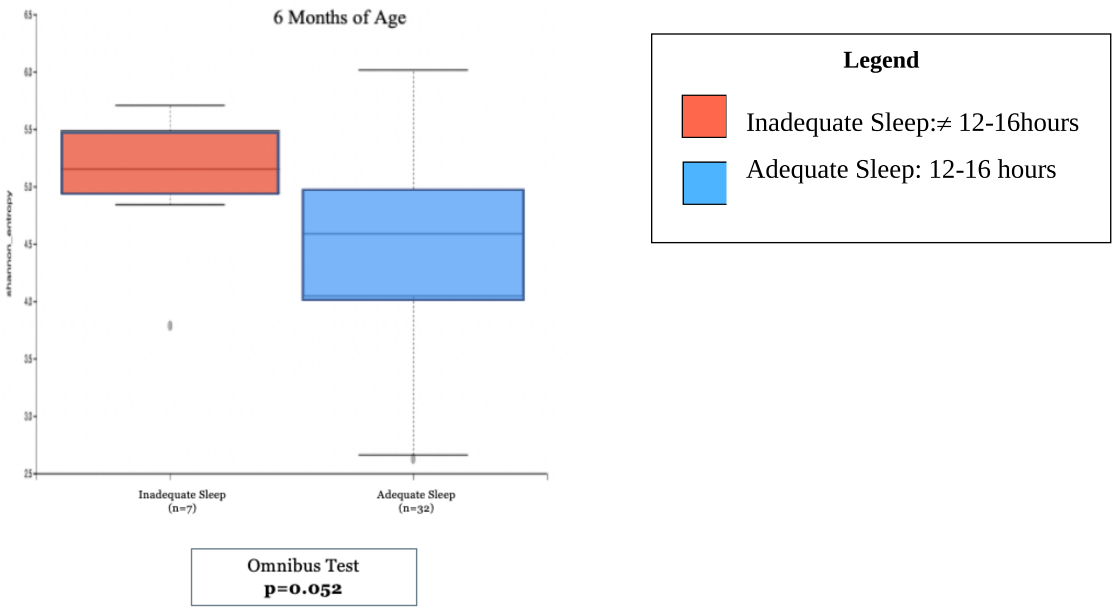


Figure 7: Shannon Diversity Kruskal Wallis Pairwise Difference for Sleep + Feeding Modality at Six Months of Age

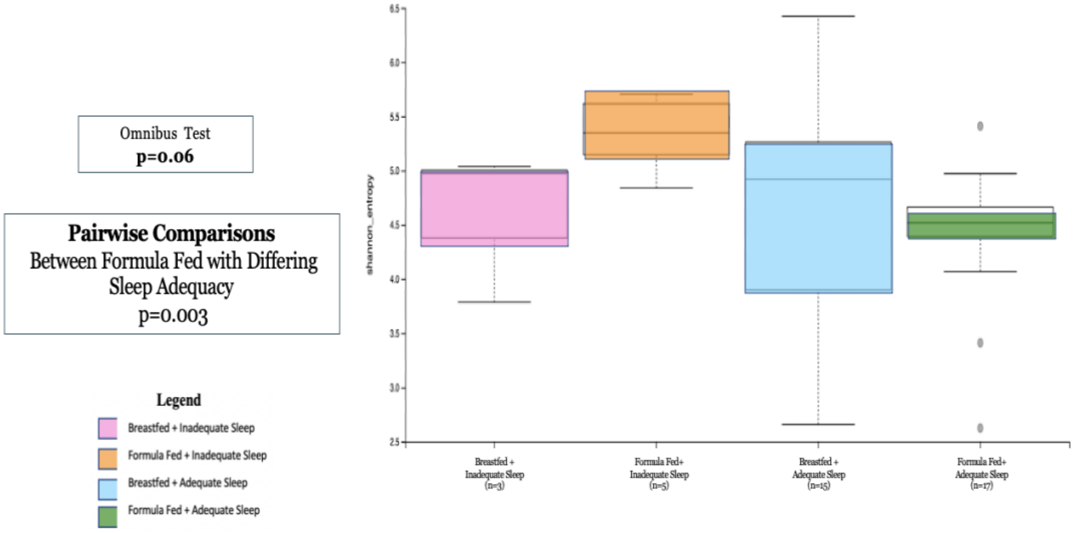
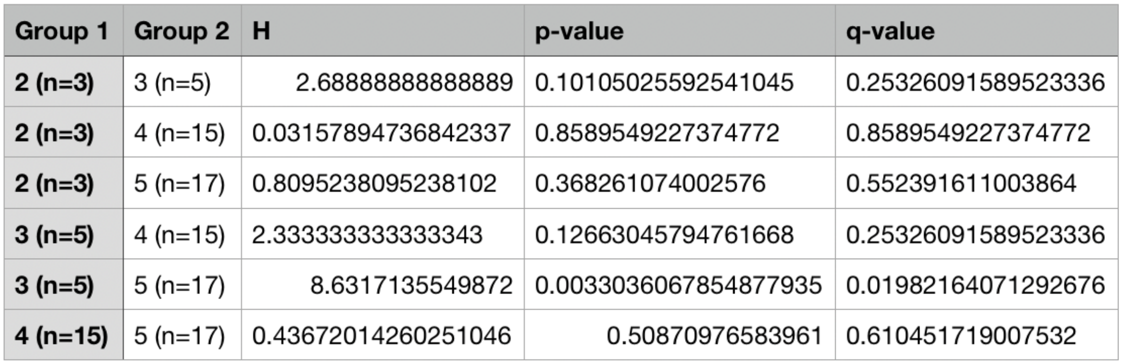


Table 4: Shannon Diversity Kruskal Wallis Pairwise Comparisons for Sleep + Feeding Modality at Six Months of Age



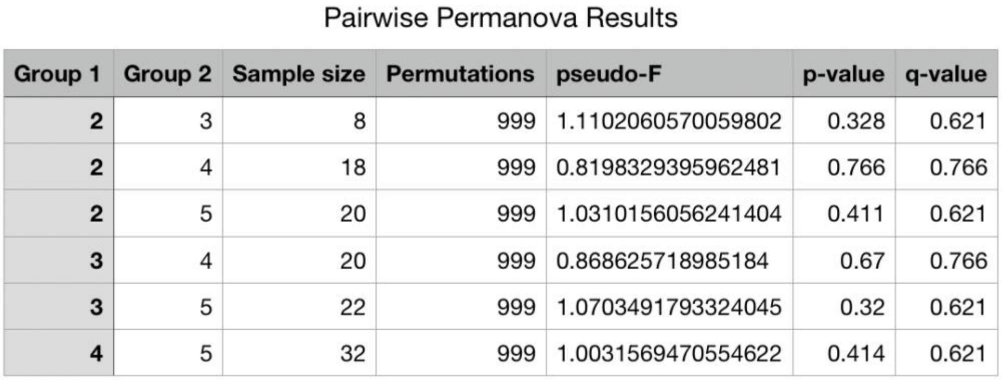
Group 1	Group 2	H	p-value	q-value
2 (n=3)	3 (n=5)	2.688888888888889	0.10105025592541045	0.25326091589523336
2 (n=3)	4 (n=15)	0.03157894736842337	0.8589549227374772	0.8589549227374772
2 (n=3)	5 (n=17)	0.8095238095238102	0.368261074002576	0.552391611003864
3 (n=5)	4 (n=15)	2.333333333333343	0.12663045794761668	0.25326091589523336
3 (n=5)	5 (n=17)	8.6317135549872	0.0033036067854877935	0.01982164071292676
4 (n=15)	5 (n=17)	0.43672014260251046	0.50870976583961	0.610451719007532

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

Beta Diversity Metrics: Bray-Curtis & Jaccard Distance

Between-sample quantitative, phylogenetic distances (Bray Curtis) in toddlerhood had no significant difference when assessed by adequate and inadequate sleep at 6 months of age (p=0.58). As observed in **Table 5** no differences were also observed for Bray Curtis-Pairwise Permanova when observing both sleep and feeding modality variables.

Table 5: Bray Curtis Pairwise Permanova Results of the Gut Microbiome in Toddlerhood Based on Combined Sleep and Feeding Categories at Six Months of Age



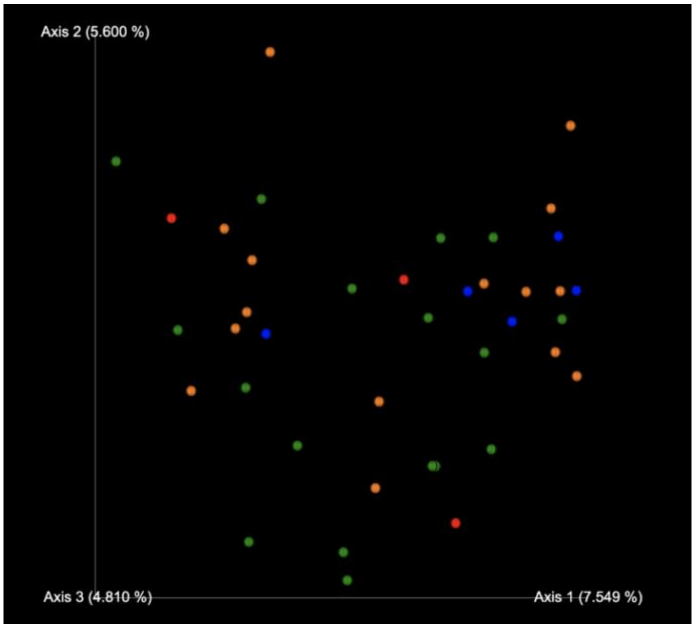
Pairwise Permanova Results

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
2	3	8	999	1.1102060570059802	0.328	0.621
2	4	18	999	0.8198329395962481	0.766	0.766
2	5	20	999	1.0310156056241404	0.411	0.621
3	4	20	999	0.868625718985184	0.67	0.766
3	5	22	999	1.0703491793324045	0.32	0.621
4	5	32	999	1.0031569470554622	0.414	0.621

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

A trending significance (p=.08, **Figure 8**) was also observed for Jaccard Distance metrics of the toddler gut microbiome based on sleep-feed categories at six months but no post-hoc pairwise comparisons suggested significant or trending group differences. Pairwise permanova results between infants receiving adequate sleep but different feeding modes (any breastmilk/formula) can be seen in **Table 6.**

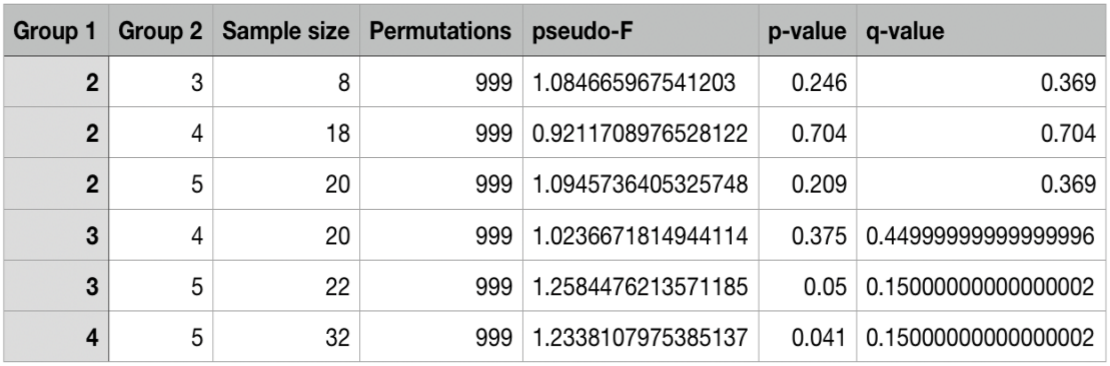
Figure 8: Jaccard Emperor Plot for Sleep- Feed Categories at 6 months of Age and Gut Microbiome Measured in Toddlerhood



Legend

🟦 Breastfed + Inadequate Sleep
🟧 Formula Fed + Inadequate Sleep
🟩 Breastfed+ Adequate Sleep
🟪 Formula Fed + Adequate Sleep

Table 6: Jaccard Pairwise Permanova Results for Sleep and Feeding Modality at 6 Months



Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
2	3	8	999	1.084665967541203	0.246	0.369
2	4	18	999	0.9211708976528122	0.704	0.704
2	5	20	999	1.0945736405325748	0.209	0.369
3	4	20	999	1.0236671814944114	0.375	0.44999999999999996
3	5	22	999	1.2584476213571185	0.05	0.15000000000000002
4	5	32	999	1.2338107975385137	0.041	0.15000000000000002

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep
:

Twelve Months of Age

Alpha and Beta Diversity Metrics for Sleep Adequacy and Feeding Modality

Alpha Diversity Metrics: Shannon's Index, Faith's PD, and Observed OTUs

Metrics used to measure quantitively for community richness (Shannon's Diversity Index) in toddlerhood showed no significance when comparing adequate (12-16 hours) to inadequate (≠12-16 hours) sleep obtained at 12 months of age (p=0.61, H=0.25). As observed in **Figure 9**, it can be observed how the infants who were breastfed and had inadequate sleep at 12 months had the lowest microbial richness in toddlerhood, and infants who were breastfed but had adequate sleep at 12 months had the highest microbial richness in toddlerhood. Despite the visual differences in Figure 10, no pairwise differences were observed between gropus (**Table 7**).

Figure 9: Shannon Diversity of the Gut Microbiome in Toddlerhood and Kruskal Wallis Pairwise Difference for Sleep + Feeding Modality at Twelve Months of Age

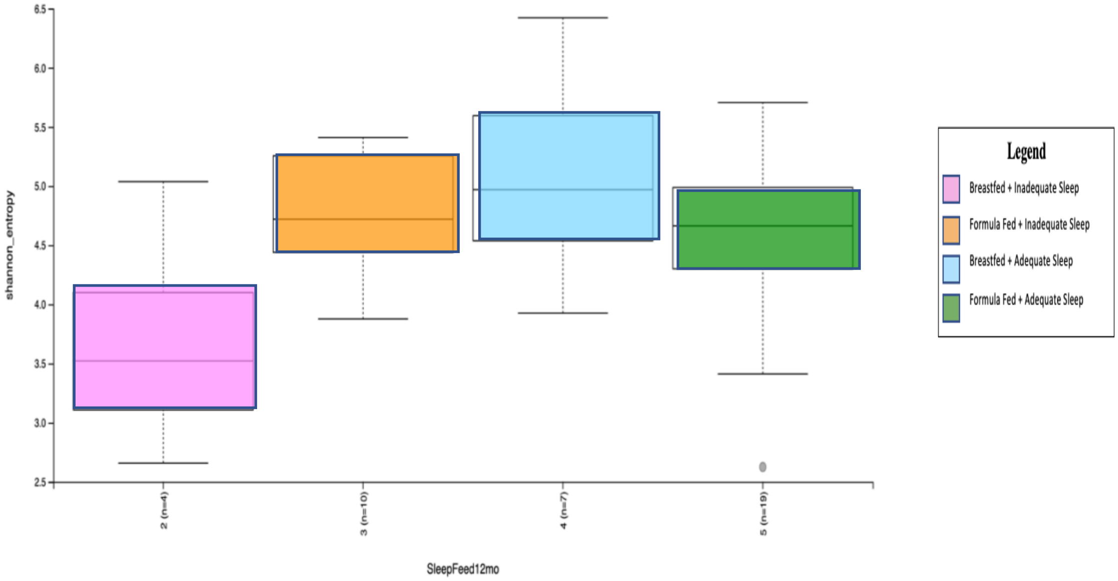


Table 7: Shannon Diversity of the Gut Microbiome in Toddlerhood and Kruskal Wallis Pairwise Comparisons for Sleep + Feeding Modality at 12 Months of Age

Group 1	Group 2	H	p-value	q-value
2 (n=4)	3 (n=10)	3.3799999999999955	0.06599205505934773	0.1979761651780432
2 (n=4)	4 (n=7)	3.5714285714285694	0.058781721355358946	0.1979761651780432
2 (n=4)	5 (n=19)	2.131578947368425	0.14429205544010074	0.28858411088020147
3 (n=10)	4 (n=7)	0.6095238095238003	0.43496716061443186	0.5219605927373182
3 (n=10)	5 (n=19)	0.05263157894736992	0.8185458083820409	0.8185458083820409
4 (n=7)	5 (n=19)	0.7025898078529735	0.40191484722585535	0.5219605927373182

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

Additional tests were performed to measure alpha diversity metrics in toddlerhood in relation to sleep and feeding practices at 12 months of age. Microbial richness (Observed OTU's, p=.03) and phylogenetic diversity (Faith's PD, p=.03; **Figure 10**) in toddlerhood were greater among children who received adequate sleep at 12 months of age. Faith's Phylogenetic Diversity measure in toddlerhood was also significantly different when compared by sleep+feed categories at 12 months of age (p=.01, **Figure 11).** Results from pairwise comparisons for Faith's PD Diversity analyses can be seen in (**Table 8**).

Figure 10: Faith's PD of the Toddler Gut Microbiome - Kruskal-Wallis Pairwise Differences by Sleep Adequacy at Twelve Months of Age

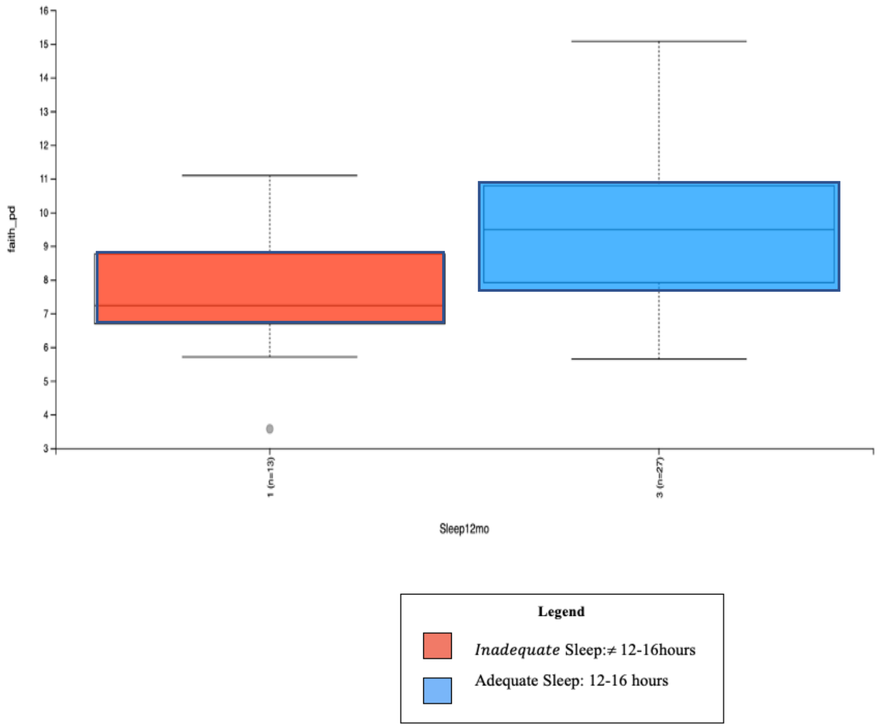
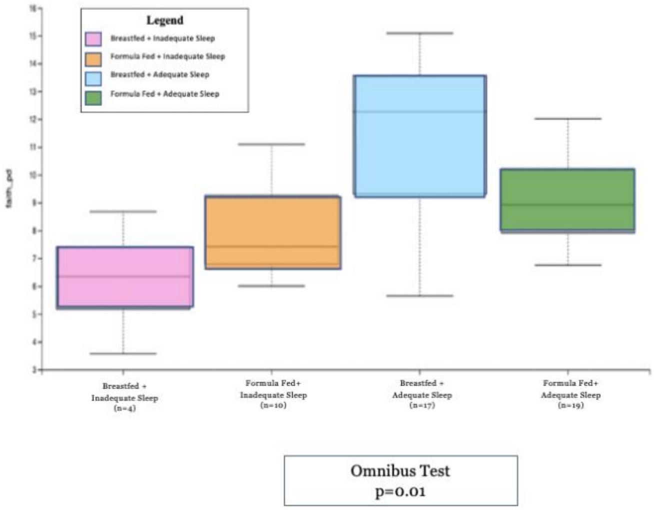


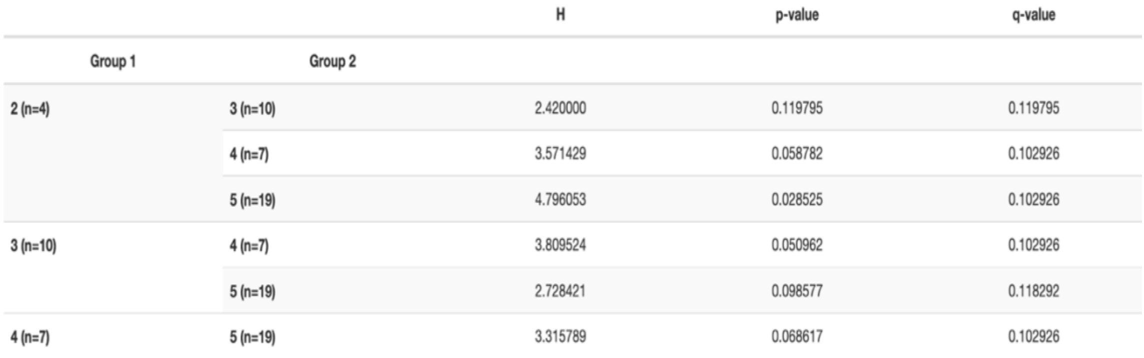
Figure 11: Faith’s PD of the Gut Microbiome in Toddlerhood - Kruskal-Wallis Pairwise Differences by Sleep Adequacy + Feeding Modalities at Twelve Months of Age



Omnibus Test
p=0.01

Table 8:

Faith’s PD Kruskal-Wallis (Pairwise) Results for Toddler Gut Microbiome by Sleep + Feeding Modality at 12 Months of Age



		H	p-value	q-value
Group 1	Group 2			
2 (n=4)	3 (n=10)	2.420000	0.119795	0.119795
	4 (n=7)	3.571429	0.058782	0.102926
	5 (n=19)	4.796053	0.028525	0.102926
3 (n=10)	4 (n=7)	3.809524	0.050962	0.102926
	5 (n=19)	2.728421	0.098577	0.118292
4 (n=7)	5 (n=19)	3.315789	0.068617	0.102926

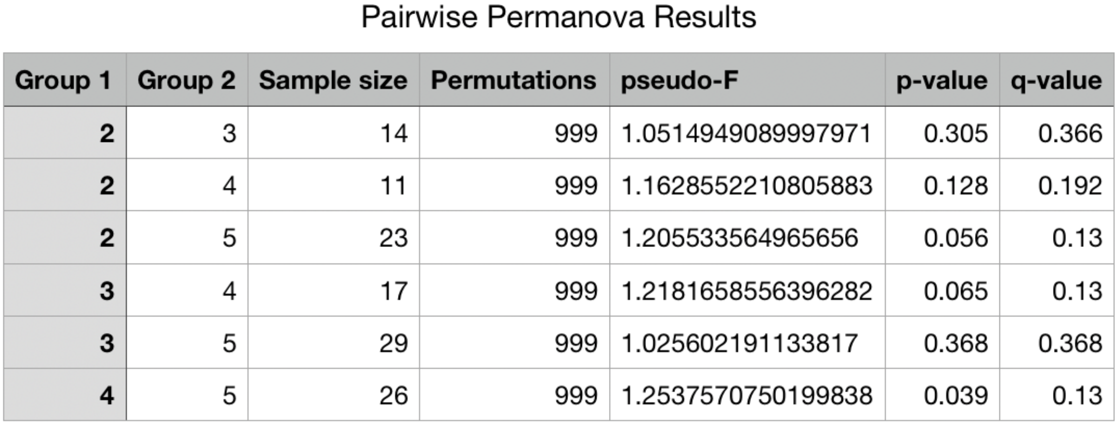
Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

<u>Beta Diversity Metrics</u>:

Bray-Curtis, Jaccard Distance, Weighted & Unweighted Unifrac Measures

A trend was observed for associations between twelve month sleep + feeding practices and the gut microbiome at 36 months of age (omnibus p=.02) using the Jaccard Distance metric, but no pairwise group differences were present after adjustment for multiple comparisons (**Table 9**).

Table 9: Jaccard Distance Pairwise Permanova Results for Sleep & Feeding Modality at Twelve Months of Age and Gut Microbiome Diversity at 36 Months of Age



Pairwise Permanova Results

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
2	3	14	999	1.0514949089997971	0.305	0.366
2	4	11	999	1.1628552210805883	0.128	0.192
2	5	23	999	1.205533564965656	0.056	0.13
3	4	17	999	1.2181658556396282	0.065	0.13
3	5	29	999	1.025602191133817	0.368	0.368
4	5	26	999	1.2537570750199838	0.039	0.13

Group Legend
2: Breastfed + Inadequate Sleep
3: Formula Fed + Inadequate Sleep
4: Breastfed + Adequate Sleep
5: Formula Fed + Adequate Sleep

Unweighted UniFrac analysis of the gut microbiome at 36 months of age also suggested group differences by sleep (p=0.04, **Figure 12**) and sleep + feeding (p=0.02, **Figure 13**) categories at 12 months. No other single (adequate sleep vs inadequate sleep) or multiple group comparisons (sleep adequacy/inadequacy + feeding modality) were significant when assessing beta diversity using Bray Curtis or Weighted or Unifrac metrics.

Figure 12: Emperor Unweighted Unifrac Plot for Total Sleep at Twelve Months and Gut Microbiome at 36 Months of Age

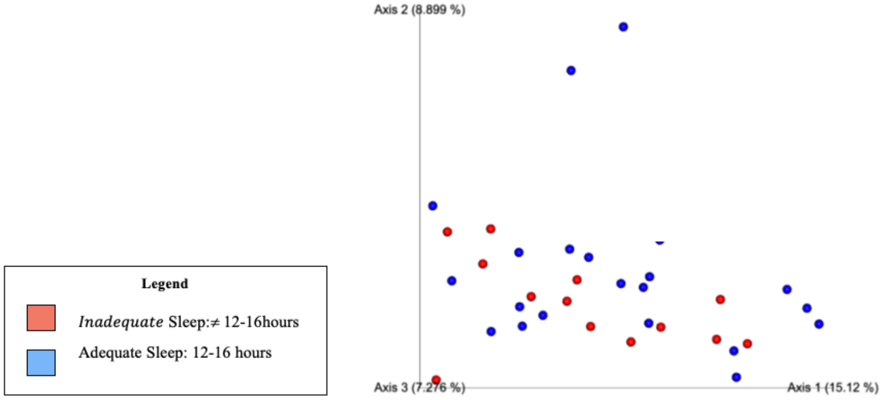
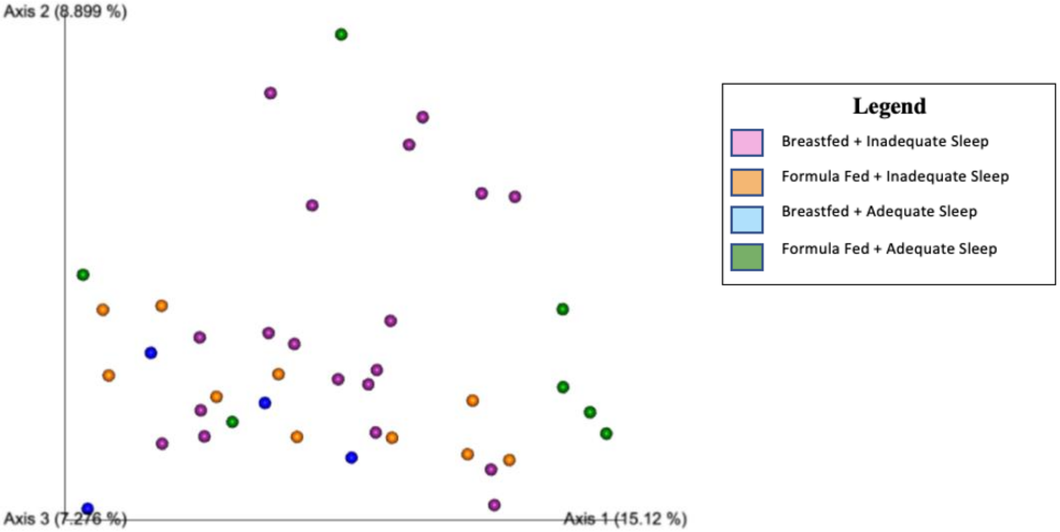


Figure 13: Emperor Unweighted Unifrac Plot for Sleep Distribution + Feeding Modality at 12 Months and Gut Microbiome at 36 Months of Age



CHAPTER 5

DISCUSSION

The purpose of this study was to research and expand the limited literature present in associating modifiable factors (sleep patterns and feeding modalities) in infancy with the gut microbiome during toddlerhood. Specifically, the emphasis was on the first year of life and how it can play a fundamental role in the establishment of the gut microbiome and how it can be then trailed into toddlerhood.

Our results varied throughout different ages during the first year of life. Both aims included exploring associations between early sleep-wake patterns alone or with feeding modalities in infancy and their influence on alpha and beta diversity during toddlerhood. It was hypothesized that infants meeting total daily sleep requirements at one, six, and twelve months, would have greater gut microbial diversity and abundance in toddlerhood then those not meeting requirements. In addition, it was hypothesized that infants receiving breastmilk and meeting total daily sleep requirements at one, six, and twelve months would have greater gut microbial community diversity and abundance in toddlerhood compared to infants that were formula fed and/or were not meeting total sleep recommendations. Overall, our results showed that infants having adequate sleep mainly at 6 and 12 months had a higher microbial community richness and abundance, although statisticial significance was not always achieved. No significant findings were observed at one month, rejecting our hypothesis for that time period. Perhaps, the first month of age may be harder to analyze, since infants are still adjusting to their new environment, and feeding practices might not yet be established, and sleep patterns may still vary. Six months is usually an age when infants start sleeping throughout the night on a regular basis and sleep patterns are more established (Stanford Children's Health, n.d.).

Research has shown how the dynamics of the infant gut microbiota are influenced by other environmental factors. Delivery mode and gestational duration have been correlated to the microbiota acquiring a greater abundance of Bifidobacterium and Collinsela at six months of age.(Dogra et al., 2015) This study's taxonomic bar plots suggest a high presence of *Firmicutes* at 36 months of age, which supports the literature on how the gut microbial community is mainly initially inhabited by Bifidobacterium, but shift occurs with age and breastmilk cessation at around 31 months of age (Dogra et al., 2015). In future analyses, it'd be of high interest to obtain fecal samples more frequently during those first 31-36 months and follow such shifts alongside sleep data collection.

Emerging, but limited, studies have hypothesized how sleep inadequacy and circadian misalignment in infancy may be potential stressors to the gut microbiota.(Parkar et al., 2019) With the results of this secondary, cross-sectional analysis, six months of age seems to be a potential window of opportunity for establishing adequate sleep patterns and feeding practices, with the intention of focusing on the gut microbiome's richness and evenness. For infants with adequate sleep duration, microbial richness later in toddlerhood was lower than those who received inadequate sleep at 6 months. Similarly, a study conducted by Schoch et al, found that actigraphy-assessed sleep habits were associated with changes in gut microbiome alpha diversity.(Schoch et al., 2022) This is the only study that evaluates sleep and the gut microbiome in infancy to date.

Studies in adults have shown how two nights of decreased partial sleep deprivation (4.25 hours vs 8.5 hours) increased the Firmicutes:Bacteroidetes ratio, leading to a greater presence of Coriobacteriaceae, which has been found to be associated with good metabolic health in the overweight and obese population(M.-H. Kim et al., 2020) and improved glucose metabolism(Liu et al., 2018) plus lower abundance of Tenericutes, which has been observed in maternal obesity(Benedict et al., 2016; *Tenericutes - an Overview | ScienceDirect Topics*, n.d.). On the other hand, another cross-sectional study placing participants in two rounds of partial sleep restriction found that the gut microbial composition was maintained, and no changes were identified.(Zhang et al., 2017) Literature is contradicting and supports a rich microbial community in adults to achieve better, optimal health but also, Lozupone et al(Lozupone et al., 2012) showed the perspective of how a high microbial richness may negatively impact an individual and in some cases be correlated to disease. Given the significant health consequences associated with microbial perturbation, it has been proposed that the first 24 months of life represent a critical developmental window for the establishment of the microbiome and greater diversity is healthy during toddlerhood.(Gaufin et al., 2018)

Feeding modalities were a key variable to assess for this study. Besides mode of delivery, literature supports the impact that early nutrition is a vital factor for early microbial composition.(*How Colonization by Microbiota in Early Life Shapes the Immune System*, n.d.) It has been shown that breastfed infants' gut microbial composition is mainly dominated by Bifidobacterium and Lactobacillus(Bäckhed et al., 2015; Rautava, 2016), while formula-fed infant microbiomes tend to be more diverse. Data from this study also illustrated higher diversity among exclusively formula-fed infants.

Since human milk bacteria composition can vary from one mother to another, the goal of this study was to see how sleep combined with different feeding modes at different months in the first year of life associated with the gut microbiome at 36 months of age. Two groups that particularly stood out across alpha and beta diversity metrics multiple times were infants who were receiving any breastmilk and having adequate sleep, and infants who were receiving only formula and receiving adequate sleep. The reason why this stood out was because even though these are differing feeding modalities, if the infant(s) were receiving adequate sleep, similarities of the gut microbial community structure were found. Perhaps, such finding can open the door for further work assessing the independent and dependent effects of early life nutrition and adequate sleep on gut microbiome development.

At six months, trending alpha-diversity differences between formula-fed groups with different sleep adequacies had interesting findings. Differences from these two groups in comparison with the rest appeared to emerge, showing greater diversity in infants receiving formula regardless of sleep adequacy. Future explorations could also look at specific trends of total amount of breastmilk consumed per feeding, and if solids were introduced before or after six months.

Strengths of the study were that mother-infant dyads were followed over the course of the first year of life and completed feeding and sleep questionnaires at multiple timepoints, allowing for multiple assessments of sleep-wake patterns and feeding practices in relation to the gut microbiome in toddlerhood. The participants in our study were all Mexican American women and infants, which is an often-overlooked child-maternal population in the microbiome literature. Limitations of the study include a small sample size (n=40, 39 at the first month since one participant's data were not collected). Additionally, the collection of the fecal sample was only at 36 months of age, which may not accurately reflect the dynamic nature of the gut microbiome during this formative period. Future analysis may benefit from collecting samples at different months of age, particularly when other questionnaires are being completed. The fecal samples were shipped within 24 hours of collection, which may have impacted the overall quality and microbial community within the samples. The analyses were limited to only observing for differences/similarities within different alpha and beta diversity metrics, but not to specific taxonomic presence of different microbes and their function.

CHAPTER 6

CONCLUSION

In conclusion, this secondary cross-sectional analysis suggests that adequate sleep patterns and feeding modalities (mainly breastmilk) during infancy may play a role in the development of a diverse gut microbial community into toddlerhood. Research is limited on how these two may be vital for gut microbial development and maturation, especially in the Hispanic population. However, the findings from this study highlight how different time stamps in the first year of life may represent important gut microbiota development milestones. The interaction between sleep-wake patterns and gut microbiome development is scarce, although this study had some limitations, the aim with these results is to demonstrate a trend for significance of the influence of sleep patterns and feeding modalities and gut microbiome community structure later in childhood. Further investigation is needed to monitor changes and potentially create a criterion for sleep adequacy during infancy to support the establishment of a healthy gut microbiome in the first year of life for long-term health.

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