Maternal microbial contributions and ontogenesis of the infant microbiome in health and behavior

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Psychology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2019

Date of final oral examination: 12/06/2019

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ABSTRACT

Over the last decade, myriad scientific discoveries have highlighted the essential role of the gut microbiota during infancy for normal development, including for nutrient extraction and metabolism, maturation of immune competence and neurobehavioral phenotypes. Microbes begin colonizing the gastrointestinal tract rapidly during vaginal delivery and acquisition continues postpartum through exposure to microbes derived from the mother, breastmilk, and the rearing environment. This dissertation utilizes a rhesus monkey (*Macaca mulatta*) model and recent advancements in gene sequencing to gain further insights into the evolved expectation for early exposure to microbes, aiming to elucidate the salience of maternal contributions to the infant's gut microbiome and to improve our understanding of the bidirectional signaling pathways in the gut-brain axis. Through a descriptive examination of the trajectory of microbiota across infancy, we first establish that the bacterial community structure of the infant gut is dynamic, gradually becoming more diverse, with the maturation of microbial community structure influenced by the transition from breastmilk to solid foods (**Chapter 2**). After characterizing the normative patterns of microbial succession, an observational study explored how the maturation of gut microbiota was influenced by maternal care, and was associated with the infant's behavior, and neurodevelopment (as determined by neuroimaging). Commensal taxa were found to vary with consummatory behavior and infant activity levels, but only the variation in the abundance of *Faecalibacterium* was significantly associated with infant temperament and neurodevelopment. Delayed acquisition of microbial community evenness and richness had physiological consequences and was predictive of slower growth trajectories and decreased neural volume at 1 year (**Chapter 3**). Lastly, the early rearing

environment was manipulated to investigate the benefits of exposure to the mother and breast milk for gut colonization and infant health outcomes. Cesarean-delivered and formula-fed infants had delayed trajectories of bacterial colonization, which were characterized by lower abundances of commensal taxa and a greater susceptibility to *Campylobacter*-induced diarrheic symptoms (**Chapter 4**). Collectively, these studies further characterized several critical windows of infant development during which the patterns of gut microbial acquisition can impact host physiology, behavioral and brain maturation, and consequently, may have important implications for understanding the role of microbes and a dysbiotic gut in neurodevelopmental disorders.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Chris Coe. Chris has been an invaluable source of ideas, knowledge, guidance, and feedback over the last 6 years. He has shown me through example what a good scientist and advisor should be, and under his mentorship, I have become more confident in my own abilities. I would additionally like to express my appreciation to my committee, Drs. Cameron Currie, Cathy Marler, Laura Hernandez, and Lauren Riters, who have been integral in shaping and refining my projects and ideas throughout this dissertation. I very much appreciate Drs. Riters and Stephen Gammie for creating a friendly environment for presenting and receiving constructive feedback on research. I also acknowledge and thank our collaborators, Drs. Mark Lyte and Greg Phillips, who managed the processing of hundreds of samples and contributed to the conceptualization of the project that became Chapter 4 of this thesis.

I gratefully acknowledge my home lab and the tireless work of my research peers, particularly, Dr. Gabrielle Lubach who keeps the lab running and was instrumental in collecting the data used within this dissertation. I owe a dept of gratitude to my academic sibling Dr. Wellington Amaral for his guidance in data analysis and copious constructive feedback. I am also thankful to the graduate students in the Biology of Brain and Behavior group, especially Caleigh Guoynes and Michelle Redinbaugh, for their scientific enthusiasm and for fostering a supportive academic community.

I would like to thank my family most profoundly- My parents, Mike and Lisé, for their love and for encouraging me to pursue my interests and to my siblings, Jake and Adrienne. Lastly, I would like to thank my partner and friend, Martin Zettersten, for his unwavering

support and his perspective, which has been truly reassuring to me at some of the more challenging times of my studies.

THESIS OUTLINE

- **Chapter 1: Introduction**

- **Chapter 2:** Descriptive examination of the maturational trajectory of the gut microbiota across the first year of life in the rhesus macaque (*Macaca mulatta*)
  - Objective 2.1: Characterize the gut microbiome of the maternal rhesus macaque over the course of lactation.
  - Objective 2.2: Describe colonization and define periods of successive changes in the infant gut microbiome.
    - Aim 1: Profile normative succession in the infant microbiome and predicted functional implications over the course of the first year of development.
    - Aim 2: Assess vertical transmission from the maternal microbiota and identify the age at which the infant resembles the mother and species-typical microbiome.

- **Chapter 3:** Neurobehavioral correlates of the composition and sequential maturation of the gut microbiota
  - Objective 3.1: Establish if the infant's bacterial profiles are associated with emotional reactivity and infant temperament at any stage across the first year of life
  - Objective 3.2: Examine if the temporal changes in mother-infant relationship are associated with shifts in the infant's gut microbiota. Specifically, if the progression toward infant independence and the initiation of eating solid foods are associated with microbial changes.
  - Objective 3.3: Delineate how variation in specific taxa and the microbial community structure may be associated with affective behavior and neural areas of the brain associated with emotionality.

- **Chapter 4:** Maternal and breast milk influences on the infant gut microbiome, enteric health and growth outcomes of rhesus monkeys (*J Pediatr Gastroenterol Nutr. Publication*)
  - Objective 4.1: Assess patterns of microbial colonization and predicted metabolic functionality in infant monkeys reared on formula by humans or by the biological mother.
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- **Chapter 5: Conclusion**

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INTRODUCTION

Early postnatal development requires an intrinsic sensitivity to environmental factors, and within mammals, there is a biological expectancy that the infant will bond with a caregiver during this period of dependency. Among primates, the caregiver is most often the mother and her presence is a direct determinant of the infant's psychological and physiological health. In addition to her time and energy resources, there is also the biological expectancy that the mother will provide and promote the initial colonization of the infant gastrointestinal (GI) microbiota. Increasing research into host-microbe interactions may provide a new avenue of investigation through which the mother impacts infant wellbeing. The initial inoculation with maternal microbes occurs during the birthing process, and is then reinforced through breastfeeding, which in addition to providing nutritional sustenance, helps to organize the sequential colonization of the infant GI tract microbiota. This phase of rapid change and plasticity within the infant gut takes place during several critical developmental windows in early life, progressing in parallel with immune and metabolic development and the establishment of the gut-brain axis, a pathway of bidirectional signaling between the GI tract and the brain. The timing of these microbial inoculations is consequently important, affecting the development and physiology of the infant early in life and subsequently gut and metabolic health and disease later in adulthood.

Beyond parental decisions about delivery mode and type of feeding, the mother may further influence the establishment of the gut microbiota through the quality of her care and warmth of maternal-infant bonding. In addition to allowing the vertical transmission of microbes from mother to infant, it is known that the supportive aspects of parental caregiving

promote neurodevelopment. Low-quality care can predispose for greater infant stress reactivity and result in dysregulated responses to environmental challenges, which in turn have been linked to differences in the composition of the gut microbiota and even to a dysbiotic and inflamed gut. Given that these complex bidirectional relationships between the gut and brain are integral to infant health and well-being, it is critical to better understand which factors are most salient and can influence the microbial populations in the infant gut during key windows of infant development.

This dissertation aims to provide further insight into this evolved expectation for early exposure to microbes, to elucidate the salience of maternal contributions to the establishment of gut microbiota, and to improve our understanding of the reciprocal brain-gut-bacteria signaling pathways. To address these questions, I took advantage of recent advancements in DNA sequencing, which allow for rapid identification of entire microbial communities. I will first describe the vertical transmission of microbes from mothers to normally developing infants and identify the critical windows of development during which microbiota structure is associated with infant physiological growth outcomes. I will then show how individual variation in patterns of colonization can be predicted by differences in maternal care and infant behavior and neurodevelopment, demonstrating the involvement of the gut-brain axis in the regulation of early life experience and the role of the gut microbiota in modulating stress reactivity. Finally, in order to better document how a delayed acquisition and/or dysregulation of the infant microbiota becomes manifest and to consider its long-term effect on infant outcomes, I will then describe patterns of microbial succession and the consequences for infant health and well-being when normal care provisions are not met, such as by substituting formula for breast milk.

Before addressing these research objectives, several caveats should be noted. Host-microbe interactions occur at numerous bodily interfaces, but this dissertation takes a more focused approach on the gut in an effort to systematically elucidate the relationships associated with the gut microbiome that contribute to infant health. On this point it is important to highlight that while microbes colonize the entire length of the GI tract, over 70% of all microbes present in the body can be found in the colon [1]. Therefore, while microbes both on and in the body can potentially influence the host, I am focusing on the predominant microbial community of the hindgut. Similarly, because the majority of microbes in the gut are bacteria, the discussion will be limited to bacterial interactions with the host, although recent work has also highlighted the importance of other microbial eukaryotes, viruses, and bacteriophages [2]. These caveats notwithstanding, the findings presented in the following chapters deepen our understanding of the gut microbiota of the young infant and the many factors that contribute to the establishment of the gut microbiome.

BACKGROUND

EARLY ACQUISITION AND DEVELOPMENT OF THE MICROBIOTA

The gut microbiota is a diverse and dynamic ecosystem, which has evolved in a mutualistic relationship with the host. It is shaped by multiple factors throughout an individual's life, but it is early exposure to microbes that may be most critical for long-term health. There is controversial evidence suggesting that exposure to maternal microbes may first occur prenatally via transplacental transfer [3,4]; however, the first major opportunity for large-scale bacterial inoculation occurs at the time of birth and in the immediate postnatal period,

when the infant is exposed to bacteria originating from the mother's vaginal, stool and skin microbiota [5]. Infants who are born through Caesarean section, bypassing this vaginal seeding, have distinctive microbial profiles that more closely resemble the skin microbiota of the mother and, if born premature, that of the Neonatal Intensive Care Unit (NICU) [6]. It should be noted, however, that while some maternal vaginal and skin microbiota strains are detected on the infant immediately following birth, the more abundant taxa in the maternal gut appear to be better able to colonize the infant gut persistently [7]. In fact, profiling the microbial content of the infant gut from birth until 2 years of age revealed a prolonged impact of birth mode, with infants delivered via cesarean section exhibiting alterations in the phylogenetic diversity of their gut community and an increased risk of gut dysbiosis [8]. These findings suggest that mode of delivery has a large impact on the establishment of the microbiota.

Though initially transitory, this first inoculum of bacteria is thought to be important for defining the successional trajectories leading to more complex and stable adult microbial communities. Following birth, the infant continues to acquire microbes from the environment and through contact with other conspecifics. Nevertheless, because infants tend to have the most social contact with their mothers, the composition of the infant microbiota continues to be heavily influenced by the mother [9]. In healthy human infants, the gut microbiota gradually shifts in diversity, acquiring a more stable composition, and the numbers of obligate anaerobic taxa increase. These shifts in the abundance and types of bacteria in the infant gut appear to largely be the consequence of breastfeeding, especially the prebiotic constituents of breast milk that support the proliferation of specific bacterial species.

Given the importance of microbiota in development, any factor that affects microbial composition during early life has the potential to influence the immune, metabolic, and brain development of an infant. Relying entirely on studies of human infants, however, it is difficult to definitively elucidate the salience of influential factors due to the inability to maintain experimental control of all variables in an independent manner. Nevertheless, besides the initial maternal inoculation occurring at birth, there is strong evidence that the infant's diet is a major determinant of the diversity and abundance of intestinal microbiota. Accumulating evidence indicates that breastfeeding and formula-feeding foster distinctive microbiotas, with the gut bacterial composition of exclusively breastfed infants characterized by a higher relative abundance of *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and genera within the Proteobacterium phylum [10]. Gram-positive *Bifidobacteria* and *Lactobacillus* in the infant gut are important inhibitors of the growth of pathogens, modulate mucosal barrier function, and do not possess proinflammatory lipopolysaccharide chains; thus, their propagation within the infant gut does not trigger full-fledged immune responses [11,12]. In contrast, the gut microbiota of formula-fed infants is more diverse, but less stable, and enriched with *Clostridium* and *Enterobacteria* [13]. These differences likely stem from the unique content of human milk oligosaccharides (HMOs) in breast milk, which constitute the third largest component after lactose and lipids. HMOs are only partially digested in the small intestine and reach the colon largely intact, where they are then fermented to produce short-chain fatty acids [14,15]. HMOs thereby have a prebiotic effect on the resident bacteria, selectively stimulating the growth and colonization of commensal strains, ultimately encouraging a *Bifidobacterium*-rich microbiota. The finding that HMOs constitute a large proportion of milk, despite being primarily

inaccessible to digestion by the infant, suggests that there is an evolved expectation that breast milk will be provided to guide and structure the gut colonization during the first year of life.

Beyond the influence of prebiotics as a stimulatory substrate, there is evidence from cultivation-based studies, as well as more recent work using 16S rRNA profiling of the bacterial community, that there is also some vertical transmission of bacteria directly from mother to infant that occurs through breast milk. The composition of this milk microbiota is dynamic, shifting from a more diverse community, including typical skin- and enteric-type organisms in colostrum, to a community characterized by less diverse flora as lactation progresses [16]. It is noteworthy that gut-associated obligate anaerobes are among the strains detected in breast milk and suggests that breast milk functions to initiate the microbial trajectory of the infant gut [17,18]. In addition to the bacteria within breast milk, the act of breastfeeding itself contributes to the continued inoculation of the infant gut. During lactation, the nipple and surrounding areolar region are in the infant's mouth introducing maternal skin-associated bacteria to the infant's oral cavity and enteric tract [18]. It is estimated that while 25-30% of the infant microbiota originates from breast milk, and an additional 10% derives from areolar skin [19].

In sum, through nursing, mothers are providing both food for their infants and a competitive advantage for the bacteria responsive to the HMOs, resulting in the continued inoculation and establishment of a commensal microbiota. This thesis further explores these relationships using non-human primates in controlled laboratory settings to more definitively elucidate the importance of breastfeeding for the maturation of the infant microbiome, and includes a descriptive analysis of nursing behavior and the infant microbiome, followed by a

controlled experiment where the influence of breast milk and formula will be directly compared.

As infants grow and begin gradually incorporating solid foods into their diet, microbial diversity of the gut has been shown to transition to a mature profile. The early microbiota progress from species that facilitate lactate utilization to anaerobic taxa involved in the digestion of solid foods and the fermentation of complex plant carbohydrates [20,21]. In healthy humans and non-human primates, the abundances of the phyla Bacteroidetes and Firmicutes become enriched and eventually dominate the composition of the bacterial community in response to changes in diet [22,23]. In humans, the acquisition of this more complex and diverse structure occurs around the end of the first year of life, and resembles that of adults around 3 years of age [20]. In addition to facilitating nutrient utilization, there is evidence that the ecological succession of intestinal microbes is responsible for calibrating the host's metabolism, educating the naïve immune system, and contributing to the neurodevelopment of an infant through bidirectional signaling in the gut-to-brain and brain-to-gut axes. Accordingly, any disruption of these normal developmental patterns prior to the attainment of a more stable adult-like gut microbiota might be expected to have a long-lasting adverse effect on health outcomes that could persist into adulthood [24].

INFLUENCE OF MICROBIOTA IN IMMUNITY AND METABOLISM

The time course of the transitioning microbiota occurs during a critical window of development and overlaps with the priming and establishment of other physiological systems. In particular, there is now extensive research in germ-free mice suggesting that early-life

bacterial colonization coincides with a delimited time period during which the immune system is permissive to microbial instruction [25,26]. This initial highly regulated exposure to commensal microbial species benefits the host by augmenting gut barrier function and enhancing protection against subsequent invading opportunistic pathogens. For example, the vertical transfer of *Lactobacilli* acquired during delivery from the mother's vaginal canal helps the infant gut maintain a low pH, limiting the opportunity for potentially pathogenic microbes to grow.

This initial colonization with commensal microbes also has a more enduring influence on the maintenance of host homeostasis. The innate and adaptive immune systems are both dependent on the colonization of the mucosal tissues of the gut with commensal microbes and are involved in regulating and/or initiating inflammatory responses. Under normal developmental conditions, these initial exposures to commensal microbes have been observed to induce mucosal antibodies and a regulatory T-cell-mediated response, which suppresses the T-helper 2 activation associated with immune-mediated and hypersensitivity reactions [27–29]. Subsequently, a potential dysregulation of the intestinal microbiota, particularly during the first year of life, is thought to bias the maturation of the immune system toward a hypersensitive or inflammatory state [8]. Dysbiotic gut microbial communities, characterized by diminished microbial diversity at 3 months of age and a decreased ratio of Enterobacteriaceae to Bacteroidaceae at 1 year of age were associated with increased sensitization to allergenic foods among 1-year old children [30]. Furthermore, these inoculations are time-sensitive. Germ-free mice that are colonized by gut microbes at three weeks of age continue to exhibit

permanent differences in immune function despite the establishment of a typical gut microbiota [31].

Gut microbiota are also known to contribute to host metabolism both directly and indirectly. One of the most important roles of gut bacteria is to catabolize dietary fibers that are not hydrolyzed by digestive enzymes and are otherwise inaccessible to the host. Gut microbial fermentation of dietary fiber produces short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate, which are essential sources of energy for the host [32]. The gut microbiota also produce vitamins and regulates xenobiotic metabolism [20]. Given these metabolic functions, it is not surprising that aberrations in microbial colonization are closely associated with (and often precede) the development of metabolic disorders, such as obesity and type 2 diabetes. Metagenomic studies have confirmed that the gut microbiota colonizing obese individuals are more efficient at recovering energy from resistant dietary fiber and more likely to promote low-grade inflammation than the gut microbiota of lean individuals [33]. Significantly, early-life antibiotic exposure has also been found to result in an altered infant microbiota, disrupted metabolism, and greater infant adiposity [34,35]. However, the salience of more subtle alterations of the gut microbial succession, such as those arising from formula feeding, on infant metabolism, growth, and susceptibility to infection is not currently well understood.

FORMATION OF THE GUT-BRAIN AXIS AND INFANT EMOTIONALITY

In addition to calibrating the host's metabolism and educating the naïve immune system, the vertical transmission of maternal microbes to offspring and the subsequent

colonization of the infant gut microbiota coincides with critical periods of brain plasticity and the development of the gut-brain axis [36]. Broadly defined, the gut-brain axis includes the central nervous system (CNS), neuroendocrine systems including the hypothalamic-pituitary-adrenal axis (HPA), neuroimmune systems, sympathetic and parasympathetic arms of the autonomic nervous system including the enteric nervous system (ENS) and the vagus nerve, and the gut microbiota [1,37,38] (see Figure 1 for detailed illustration of the gut-brain axis). This bidirectional communication network enables signals from the brain to influence the motor, sensory and secretory functioning of the GI tract, and conversely permits visceral messages from the gut to influence brain function, including the activation of the neural circuits involved in stress regulation. The gut-brain axis develops during early life and is responsive to modification by the gut microbiota [39,40]. Microbiota communicate with the host via the production of neuroactive compounds; including antimicrobial peptides, short chain fatty acids, cytokines, and vitamins, as well as most of the neurotransmitters found in the brain (i.e. dopamine, GABA, serotonin, and norepinephrine) [41]. These compounds reach the brain through circulating blood and circumventricular organs or via the vagal nerve [42]. Within the ENS, it has been demonstrated that the intrinsic primary afferent neurons embedded within the gut wall are also responsive to changes in intestinal bacterial status, and the electrophysiological properties of these myenteric sensory neurons are altered in the absence of colonizing bacteria [43,44]. The importance of this communication pathway between nervous systems becomes apparent in the high comorbidity (60%) between stress-related psychiatric symptoms, such as anxiety and depression with GI tract disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) [45,46]. Chronic GI disturbances

are also a common co-occurring clinical feature in neurodevelopmental disorders [47]. Given the bidirectionality of host-microbe interactions, it is possible that the etiology of gut-brain axis perturbations in infancy may originate at numerous levels. For instance, disruptions of the vertical transmission of bacteria from mother to infant have the potential to dysregulate the bidirectional signaling of the gut-brain axis, subsequently impacting neurodevelopmental trajectories [48]. Alternatively, perturbations in the postnatal environment could affect infant emotional reactivity and maternal-infant bonding [49], which in turn may influence intestinal physiology, as well as the exposure to microbes.

The necessity of early-life exposure to maternal microbes for the postnatal establishment of the gut-brain axis has been demonstrated in studies of germ-free mice [38]. Rodents lacking the normal commensal bacteria have been found to have altered expression of genes implicated in neurophysiological processes, including those implicated in neuronal plasticity, neurotransmission, and in the morphology of the amygdala and hippocampus [50–52]. Furthermore, they have a more permeable blood-brain barrier [53]. While it has been long appreciated that stress and the subsequent activation of the HPA axis influences intestinal microbial contents [54], there is now also evidence from studies of gnotobiotic rodents that the presence of gut microbiota plays a major role in HPA axis development and regulation. Numerous studies have demonstrated that when raised germ-free, mice [40] and rats [55] have elevated levels of circulating adrenocorticotropin (ACTH) and corticosterone following exposure to mild stress. Within the brain, expression of the glucocorticoid receptor was found to be lower in the cortex, whereas corticotropin-releasing hormone (CRF) mRNA expression was elevated in the hypothalamus of GF rats relative to specific-pathogen-free rats [40]. Given that

the glucocorticoid receptor is a glucocorticoid-inducible gene, this down-regulation in its expression may be an adaptive response to excessive levels of circulating corticosterone [55]. Significantly, both suppressed and elevated glucocorticoid levels impair brain development and functioning and are associated with the subsequent development of mood and neurodevelopmental disorders [56]. Hence, the absence of exposure to maternal microbiota during development has significant and enduring effects on brain development and HPA functionality.

Beyond the transfer of bacteria between mother and infant, maternal responsiveness, sensitivity, and the quality of mother-infant interactions are potential mechanisms through which caregiving behaviors may influence offspring development and the formation of the gut-brain axis. Maternal caregiving sets the stage for biobehavioral adaptations that become the basis of the infant's attachment style with the potential to have long-lasting influences on temperament and stress reactivity [57]. The attachment relationship an infant develops toward their caregiver and the infant's temperament have been found to be predictive of individual variation in behavior, specifically activity, emotionality, and self-regulation, in the face of an environmental challenge [58]. When care provisions are not met and caregivers are either unpredictable or insensitive to offspring needs, infants show a behaviorally and physiologically defensive response, sometimes described as behavioral inhibition. In humans, infants who experienced low-quality caregiving displayed a stress-reactive biobehavioral profile, marked by greater fearfulness to novel stimuli and more negative affect during mother-infant interactions in comparison to infants who received high-quality maternal care [59]. Infants of mothers who provided lower-quality caregiving also showed larger cortisol responses following routine care

[60]. Importantly, these psychological traits appear to be stable over development, and prolonged exposure to glucocorticoids has consequences for the neural systems implicated in stress circuitry, namely the prefrontal cortex, the limbic system, and the HPA axis [61]. In children, less secure attachment and temperament phenotypes associated with greater emotional reactivity predict the later development of anxiety disorders, depression, autism, and disorders of attention [62].

These differences in infant temperament and stress reactivity may impact both immediate and long-term changes in gut physiology. While the tight junctions in the gut epithelium and mucus layer form a physical barrier to bacteria and foreign antigens in healthy conditions, increases in glucocorticoids due to stress or dysbiosis of the microbiota can cause microdamage to the gut epithelium walls and increase permeability of the mucosal barrier [48]. Functionally, these changes translate to an increased risk for the translocation of endogenous gut bacteria to extra-intestinal tissue and consequently an induction of systemic inflammation. The importance of this relationship was first highlighted by the work of Bailey and Coe in 1999 in 6-month-old rhesus macaques [63], where it was observed that due to emotional distress, a separation of the infant from the mother at weaning age altered the composition infants' gut microbiota, increasing the susceptibility to the enteric pathogen, *Campylobacter jejuni*. More specifically, maternal separation and the accompanying increase in circulating cortisol corresponded to a significant reduction of the beneficial *Lactobacilli*. Conversely, in a rodent model exposed to maternal separation, administration of *Lactobacillus* ameliorated stress-induced abnormalities in gut function, decreased pathogenic bacterial adherence and penetration into mucosa, and reduced elevated levels of corticosterone [64]. In keeping with

these results, it was observed in several studies of breastfed human infants that microbial diversity and colonization levels of *Bifidobacterium* and *Lactobacilli* were inversely related to the amount of excessive infant crying, and furthermore, that probiotic administration of commensal microorganism may elevate colic irritability [65]. Taken together, these findings suggest that exposure to stress during the establishment of the gut microflora and even in later infancy may result in altered colonization and a predisposition to the development of gastro-intestinal diseases later in life [66].

Parent-offspring relationships are reciprocal in nature, and it may be the case that an insecure attachment type and an emotionally reactive temperament in infants also contributes to maternal caregiving quality, and impacts both vertical and horizontal microbial transfer. Many pro-social and affiliative behaviors between mother and infant, such as grooming, huddling or breastfeeding, include physical contact and can function as potential pathways for microbial transmission [67]. However, parental caregiving can be influenced by infant responses, and some research suggests that 'difficult' infants with reactive temperaments are more taxing for parents, and are consequently at greater risk of receiving harsh or less responsive care [58]. Diminished maternal care and perturbations in maternal-infant bonding are likely to impair the transmission of maternal bacteria to the infant. Additionally, the attainment of developmental milestones by the infant may be delayed, further compromising the maturation of the gut microbiome in infants exhibiting insecure attachment and a behaviorally inhibited temperament. Without a 'secure' base from which to explore their environment, insecure infants may be less independent and less willing to explore their environment [68], diminishing the receipt of microbes from their rearing environment.

Despite an awareness of the bidirectional communication pathways between the gut microbiota and the brain, the critical windows during which these concurrent processes regulate and program host homeostasis are still incompletely understood. In particular, experimental research examining the relationships between early experience, neurodevelopment and the acquisition of gut microbiota is limited in human and nonhuman primates [69,70]. As noted above, there are several studies that have demonstrated an effect of maternal and psychosocial influences on the microbiota of infant rhesus macaques, and have investigated the influence of maternal separation, gestational stress, and proximity to peers [63,71,72]. These studies, however, were largely limited to monkeys over 6 months of age, or were limited to several bacterial taxa due to the use of traditional culture-based methodologies. Given that microbial dysbiosis earlier in life seems to have more significant developmental consequences, an evaluation of the successive colonization of the GI tract starting at delivery, followed by a longitudinal assessment of infant developmental outcomes is therefore necessary to further characterize the role of microbiota establishment and maturation in health and disease.

OBJECTIVES

In light of the limitations associated with interrogating these questions in humans, the objective of this dissertation is to use the infant rhesus macaque as a model to generate further insight into the early exposure to microbes, to elucidate the significance of maternal contributions to the establishment of the microbiota, and to improve our understanding of microbiota-gut-brain signaling. The rhesus monkey has been widely used in biomedical research

due to its genetic, physiological, and neurodevelopmental similarities to humans [73–75]. Non-human primates are uniquely suited to longitudinal studies of the microbiota as the developmental time course is fairly protracted, extending over a year, yet environmental influences and early experiences can be largely controlled. While specific taxa may differ between human and non-human primates, the general principles of host-microbe interaction are likely to be conserved across primate species and the associated microbial communities [76], allowing insight into potential mechanisms that are important in shaping these exchanges. Understanding these relationships from an evolutionary perspective is important given that host-microbe relationships are presumed to have evolved in environments where they provided health and fitness advantages, and modern human populations occupy dissimilar environments and experience different selective pressures [77].

AIM 1 of this dissertation is to characterize the transmission and composition of healthy microbiota over the first year of development. Use of a rhesus monkey model allows for control over living conditions and the diet consumed by both the mother and the maturing infant at the time of weaning from breast milk. Due to ethical limitations of human studies, the fate of vertically acquired strains is largely unknown [78]. Newborns receive bacterial transmission from the mother at the time of birth and also through breast milk, and this continued contact with the mother and the variable ages of human infants at the time of weaning from breast milk or formula makes it difficult to determine the degree to which vertically transmitted microbes become permanent colonizers. However, the husbandry practices used to care for and raise rhesus monkeys allow for a more controlled transition that is scheduled at the age of natural weaning from the mother, with the transfer of the weanlings into peer housing at 6-7

months of age. In Study 1, the mother-to-infant vertical transmission of microbes was tracked during the initial phases of nursing and the permanence of the maternal influence was then assessed by following microbial communities in the same infants after they have been rehoused to small social groups and are now consuming solid foods exclusively. In order to investigate the role of microbial succession in infant physiological development, as well as to generate predictions of the microbial influence on metabolic functioning, markers of gut microbiota maturity and diversity were additionally evaluated in relation to infant growth trajectories. Once the major microbial transitions have been described, the subsequent chapters characterize other aspects of maternal behavior or the infant's development that influence the microbial succession.

The objective of **AIM 2** is to provide insight into the complex interplay of gut microbiota colonization, infant temperament, and early life experiences in the development of the gut-brain axis and calibration of stress reactivity. Despite growing evidence linking gut microbiota composition to behavior and neurodevelopment, few studies to date have examined the influence of maternal-infant interactions and infant emotionality on the gut-brain axis in non-human primates. To explore these interrelationships, two experiments were conducted. In the first, rhesus infants were followed over the first year of life during which microbial profiles were examined and infant emotionality and development were documented through standardized behavioral assessments and observations of mother-infant interactions. Because infant temperament has often been found to be a stable trait, it was hypothesized that differences in community structure would emerge and persist across development in association with differences in emotional reactivity. In the second phase of this study, structural magnetic

resonance imaging scans of the infant brain were acquired at 12 months of age to investigate whether the infant gut microbiota were predictive of overall brain size, as well as the volume of selected regions that have been identified previously as linked with emotionality. A better understanding of the relationship between gut health and affective states and neurodevelopment in early life is a first step toward identifying the antecedents of neurodevelopmental disorders.

AIM 3 employed an experimental manipulation to investigate the consequences of interrupting the typical microbial transmission and influence of breastfeeding. In humans, C-section deliveries and formula feeding are commonplace occurrences that perturb the typical colonization of the neonatal gut. Both C-section deliveries and formula feeding have also been associated with an increased risk of a number of inflammatory and immune-mediated conditions, including food allergies, asthma, and atopic dermatitis, as well as increased risk of metabolic diseases, such as obesity and type 1 diabetes [6,79,80]. It has been suggested that the protective mechanisms provided through vaginal birth and breastfeeding are in part explained by their role in the establishment of the infant gut microbiota, specifically the targeted growth of commensal species [81,82]. With the third study, the goal was to obtain further evidence that gut bacteria may be a mediator between early feeding regimens and infant health outcomes. To replicate conditions in which maternal microbial transfer is interrupted in humans, the gut microbiota of rhesus infants that were born vaginally and mother-reared was compared to the microbial profiles of infants delivered via cesarean-section and human-reared with formula feeding over the first 2 months of life. The rearing environment was hypothesized to be predictive of distinctive microbial compositions, and it

was predicted that the gut of human-reared infants would be characterized by fewer species-typical commensal taxa. Because the time course of this assessment overlaps with critical periods for growth and susceptibility to diarrheic disease that can occur in young infants, the metabolic and health consequences of deviating from the normative microbial trajectory were additionally evaluated. Identifying differences in the gut microbial composition as a function of early life experience is a necessary first step to better understanding the health disparities associated with delivery and feeding modes.

To conclude, mutualistic bacterial strains within the gut have evolved alongside their mammalian hosts, and reveal symbiotic and functional linkages between the host and its surrounding environment [83]. Our evolutionary history has been intimately connected with that of our gut microbiota. However, modern hygiene, technical advances, and medical care and antibiotic use have resulted in an increasingly sterile society, and the indiscriminate of loss of diversity of both commensal and pathogenic microbes [84]. This loss of bacterial diversity seen in the Western countries has been hypothesized to have resulted in large-scale changes in the transmission and composition of gut microbial communities [85], and in many cases, such as premature birth, ultimately contributes to an aberrant colonization of the neonatal intestine. Although plasticity during critical windows of development can allow for greater flexibility in adapting to environmental conditions, it also creates a vulnerability when biological expectations are not met, and that plasticity leads to a maladaptive response. It is likely that diminished exposure to maternal sources of microbes is a significant contributing factor to our increasing susceptibility to infectious and developmental diseases, especially in the context of certain parental decisions about early feeding [5,86]. This dissertation aims to further

characterize the critical windows of microbial exposure and development and to elucidate the salience of maternal caregiving on the establishment of the infant gut microbiota. In addition, it will explore the subsequent influences of microbial colonization that reflect the infant's growth and emotional temperament, which contribute to both gut health and other developmental health outcomes. A greater understanding of these factors is a critical forerunner to realizing the potential of modulation of the gut microbiota with prebiotic and probiotic supplements as a therapeutic and preventative strategy for developmental disorders.

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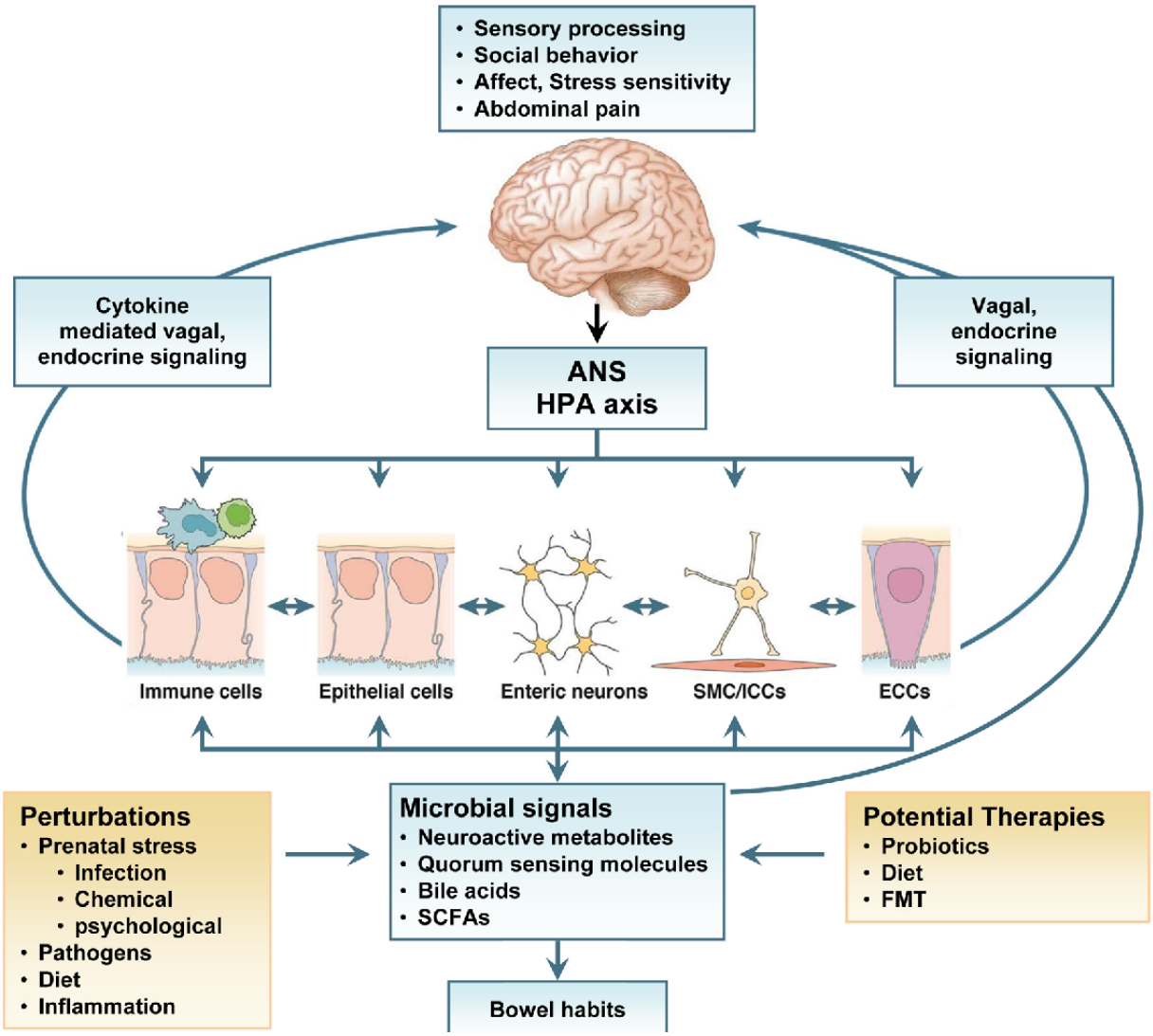

Figure 1. (Image Source: Mayer, Padua, & Tillisch; 2014) [87]. The gut-brain axis is a bidirectional pathway of communication between the central nervous system (CNS) and the enteric nervous system (ENS) of the body. It involves direct and indirect pathways between peripheral intestinal functions and affective and cognitive regions of the brain. The gut-brain axis additionally encompasses neuroendocrine and neuroimmune systems, including the hypothalamic pituitary axis (HPA), and the autonomic nervous system (ANS), including the ENS and the vagus nerve. The gut microbiota communicates with the axis through the production of short-chain fatty acids (SCFA), vitamins and cofactors, and neurochemicals, as well as through the metabolism of bile acids and xenobiotics. Under stress or disease conditions, intestinal dysbiosis impacts gut physiology and function and increases the permeability of the intestinal barrier, which can lead to inflammation.

Chapter 2: Descriptive examination of the maturational trajectory of the gut microbiota across the first year of life in the rhesus macaque (*Macaca mulatta*)

ABSTRACT

Background: Exposure to maternal microbes during delivery and early infancy provides the initial foundation for healthy gut microbial colonization and facilitates immune maturation and host metabolism. Alterations in the normal colonization of intestinal microbes during early life have been associated with pediatric disorders and increased risk for disease in adulthood. In order to better understand how bacteria within the gut interact with other microbes and with their infant host to contribute to better health outcomes, a deeper understanding of microbial succession in the infant is needed.

Method: Rhesus monkeys (*Macaca mulatta*) were utilized to characterize the infant intestinal microbiome composition and to document the transmission, progression, and eventual maturation of the gut microbial community. Rectal swabs and growth, including anthropometrics, were longitudinally collected from infants at 2-month intervals across the first year of life and rectal swabs were collected from mothers during the nursing period. Fecal microbial community structure and diversity were determined by gene amplicon sequencing and taxonomic classification of the V4 region of the bacterial 16S rRNA gene.

Results: In contrast with the maternal gut microbiome, the infant microbiome was dynamic and distinctive microbial signatures were detected in the gut microbiome composition at each sampling time point, resulting in developmental changes in the predicted metabolic functioning of these bacteria. Cessation of breastfeeding and transfer to peer-housing were major factors in driving the maturation of the infantile microbiome to a more adult-like composition and corresponded with significant increases in taxa richness and diversity. However, correlations in *Campylobacter*, *Clostridium*, and *SMB53* abundances between mother and infant persisted

following infant weaning and cohousing with peers, suggesting an enduring influence of the vertical bacterial transmission from mother to infant. The taxonomic composition of the infant microbiome at 12 months of age was still distinct from that of the mother; however, PICRUST analysis revealed that the predicted functioning of metagenomic pathways had reached a mature stage. Individual differences in gut microbiome maturation corresponded to developmental outcomes; infants progressing to a more diverse and even microbial composition, and hence mature profile, had faster growth trajectories.

Conclusions: The immature gut of the infant rhesus monkey is complex and highly dynamic, and microbiome maturation is a not a random process. Though there are distinct differences in the taxonomic composition of the gut microbiota of the rhesus infant as compared to reports in human infants, our findings further support the rhesus monkey as an informative model for research on gut development, which can enhance our understanding of the acquisition of the infant microbiome and establishment of a healthy host-microbiome relationship.

What Is New:

- In contrast to adults, the diversity, richness, and structure of infant gut microbial communities vary dramatically during the postnatal period.
- The cessation of breastfeeding and transition to housing with peers in social groups were major factors driving the maturation of the infantile gut microbiome and the predicted metabolic activity to a more adult-like profile.
- Some aspects of the commensal bacterial colonization were dissimilar to patterns reported for human infants. Rhesus infants exhibited much lower abundances of *Bifidobacterium*, and the levels of *Lactobacillus* were enriched at an older age following the switch to solid foods at weaning.
- During the nursing period, more mature gut microbial profiles with a more even taxonomic composition were predictive of faster growth during the first year of life.

INTRODUCTION

The acquisition and maturation of the infant gastrointestinal microbiome are key to establishing host-microbiome symbiosis and are an integral aspect of normal developmental and behavioral health. The compositional structure of gut microbiota and metabolites that are produced is dynamic across the lifespan, and is shaped by many factors, including host genetics, age, sex, and diet [1–3]. Recent studies suggest that the composition and functional potential of the microbiome undergoes transitional stages that parallel dynamic periods in brain development and metabolic programming, suggesting that this microbial succession has developmental relevance [4]. In keeping with this perspective, signs of a microbial dysbiosis are associated with several neurodevelopmental disorders, including autism spectrum disorder, and there is also evidence that dysbiosis has a mediating role in the later emergence of metabolic disease in adulthood [5–8]. However, while the importance of host-microbiome relationships has been well-established, the mechanisms, transmission routes, and timeline of the establishment of these infant-colonizing microbes are still poorly understood. This gap in knowledge can be attributed in part to limitations imposed by the use of studies using human infants, which usually prohibit allocation of infants to specific diets, and the challenge of eliminating confounding factors such as socioeconomic conditions, cultural factors, and antibiotic usage [3,9].

Before links between the configuration of the infant gut microbiome and developmental outcomes can be established, a more comprehensive assessment of the microbes vertically transmitted from mother to infant is needed. While the mammalian microbiota are partially acquired horizontally through environmental and social interactions, vertical transmission of

microbes from the mother to the offspring is the primary mode of acquisition in early life [10]. The accepted dogma has been that the human fetal environment is sterile, however recent studies have suggested that some maternal transmission of microbes may begin in utero [11]. While there is currently not enough scientific evidence to support the existence of a viable microbiome within the healthy fetal compartment [12,13], it is clear that extensive microbial colonization occurs soon after birth. Maternal microbes originating from the gut have proved to be particularly persistent in colonizing the gastrointestinal (GI) tract of the infant and are ecologically better adapted than bacterial strains acquired from other body locations [14].

In addition to gestational age at birth, as well as antibiotic use by the mother and young infant, delivery method and feeding mode are major determinants of the initial microbial colonization of the infant GI tract [15]. In contrast to human infants born by Caesarean delivery who develop a microbiome resembling that of skin, natural delivery exposes the infant to both the vaginal and intestinal microbiota, including the commensal taxa *Lactobacillus* and *Bifidobacteria* [16,17]. Microbial seeding continues after birth and is reinforced through maternal contact and the infant diet. The contributions of breastfeeding are two-fold; breastmilk is both a source of viable bacteria and contains oligosaccharides, which are the preferred prebiotic substrate for several commensal taxa acquired during delivery [18,19]. In human infants, there is evidence that the gut microbiota continue to develop throughout infancy and childhood, becoming more stable, diverse, and resembling that of an adult around the age of 3 years. However, beyond general descriptions of increasing complexity and stability within the infant gut microbiome with age, there is yet to be a comprehensive assessment of the long-term impact of these maternal microbial contributions, specifically the degree to

which maternal taxa permanently colonize the infant and persist in the absence of continued exposure to the mother.

To generate further information on these questions, a rhesus macaque model was employed to investigate the relationship between the mother's gut microbiome and the development of the infant gut microbiome. Rhesus monkeys are an appropriate animal model due to similarities in neurodevelopment, behavior, and metabolism [20,21], and with this study, rhesus macaque infants are additionally established as a useful model for examining the microbial succession within the infant gut. Sampling was conducted at birth, 2 weeks of age, and then at 2-month intervals throughout the first year of life, spanning two critical windows of development when the microbial community could maximally influence metabolism, growth, and neurodevelopment. Because the maternal microbial reservoir constitutes the primary source of infant-colonizing microbes, the bacterial composition of mothers' gut was first characterized from the time of birth to around six months postpartum in order to detail the role played by vertical transmission in the acquisition and establishment of the infant gut microbiome. Other studies of adult macaques have found that, like humans, the GI tract microbiome is stable and dominated by Bacteroidetes, Firmicutes, and Proteobacteria [22–24], but the microbial structure and community of lactating rhesus females had not been described previously.

Once the normal microbiome of the adult female was determined, the gut microbiota community succession and the microbial signatures from birth to one year of age were described in the infant. Rhesus infants are typically weaned from their mothers at around 6 to 7 months of age, when husbandry practices at this facility then transfer the weanlings to small

social groups with other peers, providing a unique opportunity to evaluate if there is an enduring influence of the mother that persists after the older infants are housed in another context. The possibility of sex differences in microbiome trajectories was also explored given that previous research found the sex of the infant to be predictive of the nutrient and hormonal composition of maternal milk [25,26]. In addition, there have been previous reports of sex differences in social behavior [27,28], which might influence infant exposure to both maternal and environmental sources of microbes. Finally, in order to investigate whether the patterns of microbial succession were predictive of metabolic function and growth, the maturation and diversity of the microbiome community structure was evaluated with respect to infant growth trajectories. Signs of a dysbiotic gut early in life were hypothesized to be predictive of diminished growth due to the role of the gut microbiome in host and nutrient metabolism. This characterization of the microbial colonization and succession provides the groundwork for future research on gut microbiome development both in animal models and in humans, including on factors that change microbial composition, as well as for studies of the influence of the gut microbiome on infant immune function, physiology, and neurocognitive development.

METHODS

Subjects. Infants were generated from healthy, multiparous rhesus monkeys (*Macaca mulatta*) at the Harlow Center for Biological Psychology. Both mothers and infants were part of an established breeding colony, which is housed indoors under controlled conditions. Only healthy mothers and infants were included in the study. Animals were excluded from further sampling if they developed diarrheic symptoms that required treatment with antibiotics. Mothers and

older infants were fed a standardized diet of laboratory monkey chow (PMI Nutrition International, Richmond, IN; see Supplemental Table 1 for Diet Composition) that was supplemented with fresh produce and water was available *ad libitum*. Mothers and infants were sampled within several days of delivery (average 1.69 ± 0.38), 2 weeks and then at 2-month intervals until infants reached one year of age (See Supplemental Table 2 for Subject Descriptives). Samples were collected between 0930 and 1130 to control for diurnal variation in microbial community structure. Infants were either housed only with their mothers, or with an additional adult female and her infant, until weaning which occurred between 6 and 7 months of age. After weaning, infants were placed in a social group of 3-4 similarly aged monkeys. Infant growth was evaluated from birth to 12 months of age through the trajectory of infant weight gain and the change in the length from the crown of the infant's head to its rump; an approximation of height. All animal husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Isolation and Sequencing of Genetic Material. Rectal swabs were obtained using BBL CultureSwab Collection & Transport System swab/tube (BECTON Dickinson, Cockeysville, MD) and then stored at -70°C until extraction. DNA was isolated from rectal swabs using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol. Extracted genomic DNA were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and underwent polymerase chain reaction (PCR) to amplify the V4 hypervariable region of the bacterial 16S rRNA gene using V4 region specific primers (515F-806R). Barcoded amplicons from each sample were sequenced using the MiSeq Platform (Illumina, San Diego,

CA) per manufacturer's guidelines by the Institute for Genomics and Systems Biology at the Argonne National Laboratory (Argonne, IL).

Bioinformatics and Statistics. The unmerged forward and reverse reads were imported into QIIME2 version 2018.11, a plugin-based system that wraps other microbiome analysis methods. Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with the DADA2 (q2-dada2) analysis pipeline to detect and correct observed amplicon sequence variants, including chimeric sequences. Amplicon sequence variants were aligned with mafft (q2-phylogeny) and were used to generate a phylogenetic tree. Diversity metrics were estimated using q2-diversity after samples underwent rarefaction, whereby sequences are sampled to a depth of 5000 sequences per sample without replacement and samples with fewer sequences were excluded from all downstream diversity analyses. Measures of microbiome dissimilarity, or Beta Diversity, were generated with weighted Unifrac analysis, which considers both sample phylogenetic distance and abundance to compare underlying microbial community composition. Specifically, sample similarity was determined using a phylogenetic tree to calculate the sum of "unique" branch lengths, which is then divided by the sum of "shared' branch lengths [29]. Weighted Unifrac weighs these shared nodes based on the relative abundance of each operational taxonomic unit (OTU) to calculate distance matrices, which are then used to perform principal coordinate analysis (PCoA) in order to assess community architecture. β-diversity was additionally analyzed using the Jaccard distance index in the Vegan R package [30], which evaluates the presence/absence of taxa and does not take relative abundance into consideration. Differences in bacterial community

structure were assessed using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. In order to test the homogeneity of group dispersion, BETADISPER [30] was used to calculate the average distance of group members to the group centroid or spatial median in a multivariate space. To examine the difference in bacterial communities between time points, alpha diversity metrics were employed to evaluate microbiome richness and evenness, including observed OTUs, Pielou evenness, and Faith's Phylogenetic Diversity.

In order to take full advantage of the largest possible N and to also evaluate the change within individual subjects, diversity metrics were evaluated both cross-sectionally and also longitudinally among subjects with near-complete sample collection. Longitudinal analysis was conducted using Q2-longitudinal, a software plugin for QIIME 2 [31], to evaluate temporal and pairwise changes in Faith's Phylogenetic diversity estimates and weighted Unifrac distances using linear mixed effects modeling with the Benjamini-Hochberg false discovery rate (FDR) correction. Specifically, components of variance were used to estimate the between- and within-subject intraclass coefficient for each microbiome measure, and the change in both magnitude and direction in the first principal coordinate axis (PC1) was then computed for each subject between the first and last samples. Temporal change in the mothers' microbial profiles was evaluated from the 2-week timepoint to 6 months, and infants were evaluated starting at 2 weeks and ending at 12 months of age.

Taxonomic classification was performed using a Naïve Bayes filtered classifier (q2-feature-classifier) trained on the Greengenes database 99% OTUs, version 13_8. All downstream analyses of taxonomic composition were performed at the phylum and genus level and used nonparametric analyses due to the descriptive aim of the study and non-normal

distributions at each age point. The Kruskal-Wallis and Mann-Whitney tests were used to compare the relative abundances of gut OTUs between timepoints and groups. And, Spearman's correlation coefficient (denoted by *r*) was used to estimate associations between infant growth trajectory and genera abundance, and between matched maternal and infant samples given the skewed distribution of the bacterial abundances. In order to evaluate vertical inheritance, taxa were also analyzed at the species level, but only a small proportion of OTUs were identified. *P*-values were adjusted by false discovery rate (FDR) correction.

Finally the functional profiles of the bacterial communities were explored using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [32], which uses the presence and abundance of gene families identified through closed-reference OTU picking to estimate the composite metagenome. Functional composition profiles based on the Kyoto encyclopedia of genes and genomes (KEGG, http://www.ncbi.nlm.nih.gov/pubmed-/22080510) were generated at levels 1 and 2 in KEGG Orthology (KO) hierarchies. The linear discriminant analysis (LDA) effect size from the LEfSe [33] software package was used to identify which biologically informative features in relative abundance of microbial taxa (minimum ≥ 1% relative abundance in at least one sample) or metagenome content distinguished time points from one another. An effect size threshold of 2 (on a log10 scale) was used and all categories with a significant LDA score were further analyzed with the Kruskal-Wallis test. In order to limit the number of comparisons, LEfSe analyses were reserved for samples from early lactation (2 weeks), late lactation (6 months), and samples collected several months following weaning (12 months). *P*-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg FDR correction [34] .

RESULTS

Sequencing Summary. A total of 13,243,436 reads were obtained from 269 samples collected from mothers and their infants, with a range of 1,016 to 85,998 reads per samples (mean reads = 25,573 ± 9,631). Samples with a low read count (<5000 reads), and sequence variants appearing in two or fewer samples were filtered, based on the suspicion that these may not represent real biological diversity but rather PCR or sequencing errors (such as PCR chimeras), resulting in 3,188,750 (24.08%) sequences being retained in 263 (97.8%) of samples. Rarefaction curves were generated and inspected prior to the subsequent analyses to determine whether sampling depth was sufficient to accurately characterize the microbiome (Supplemental Materials: Figure 1).

Microbiome of Mothers. Because the maternal gut is a primary source of the microbes colonizing the infant gut, it was necessary to first characterize the bacterial profile of the adult female rhesus monkey and to establish the degree to which this profile varied over the course of postnatal care. Samples were evaluated in two ways; cross-sectionally at birth, 2 weeks, and at 2-month intervals through 6 months postpartum, and longitudinally from 2 weeks to 6 months of infant age due to some samples missing on the day after birth (See Table S2 in Supplemental Materials for sample descriptives). When examined cross-sectionally, the gut microbial community structure varied significantly between timepoints across the 6-month lactation period (PERMANOVA: pseudo-F=2.04, p= 0.001 using weighted UniFrac distance metrics; Figure 1A). Specifically, inter-individual differences in sample distances taken at 6

months postpartum were significantly smaller than those at other time points (BETADISPER: $p<0.001$; Figure 1B). However, LME first distances models were then calculated from the 39 subjects with 3 or more microbial measures collected in the postpartum period and applied to weighted UniFrac distance matrices to examine temporal changes in each subject's beta diversities. The results indicated that there was no longer an effect of time on rates of transition of phylogenetic and abundance within the microbiome (REML Weighted: $p=0.183$), which suggests that any differences seen cross-sectionally are the result of individual variation. The overall alpha diversity within sample diversity was relatively similar in mothers over the lactation period; there no significant differences in Faith's Phylogenetic Diversity, Observed OTUs, or Evenness (Kruskal-Wallis: $p=0.08$, $p=0.098$, $p=0.628$, respectively; Figure 1C).

When evaluating the relative taxonomic abundance of the maternal rhesus microbiota, Firmicutes were consistently the most abundant phyla (61%), following by Bacteroidetes (28%) and Proteobacteria (5.8%). Within these phyla, the most abundant bacterial genera were *Prevotella* (20.2%), *Lactobacillus* (13.2%), *Streptococcus* (8.2%), *Flexispira* (4.3%), *Blautia* (2.4%), *Megasphaera* (2.4%), *Roseburia* (2.1%), *Oscillospira* (2.1%), *Dialister* (1.8%), *Ruminococcus* (1.8%), *Coprococcus* (1.6%), and *Faecalibacterium* (1.3%) (Figure 1). These communities are consistent with known microbiota present in the rhesus monkey gut [24,35].

Overall, there were no significant and enduring changes in the abundance of microbes constituting at least 1% of the maternal microbiome over course of lactation (Figure 1D). Although Firmicutes abundance was stable, the genus *Faecalibacterium* did increase steadily from the time of birth to 6 months postpartum (<1% at birth to 1.7% at weaning; FDR p=0.046). Bacteroidetes abundance also varied slightly, with levels decreasing to 24.5% during peak

lactation (2-4 months) before recovering around the time that the infant was weaned (FDR *p*=0.069). Bacterial taxa assigned to the phylum Spirochaetes, especially the genus *Treponema*, were diminished at the time of birth and at 2 weeks postpartum (FDR *p*=0.016), whereas taxa belonging to the phylum Actinobacteria were found to be more abundant; however, these differences were not significant following correction for multiple comparisons. Putative functional profiles of the microbial communities were then generated for all samples using PICRUSt. In keeping with the general consistency seen in microbial taxonomy, there were no significant differences in predicted metagenomic function when evaluated at Level 2 of the KEGG pathway (Supplemental Table, Supplemental Table 3). This temporal stability observed in the microbial profile of the rhesus mother follows reports made in human mothers [36] and suggests that additional factors beyond exposure to the maternal gut microbiome are responsible for the microbial succession of the infant gut.

Microbiome of Infants. In contrast to the relative stability of the maternal gut microbiota, quantitative taxonomic profiling revealed that the diversity, richness, and structure of infant gut microbial communities vary dramatically over the course of the first year of life. When examined cross-sectionally, samples collected at birth, 2 weeks and at 2 through 12 months postpartum indicated that there were significant differences in gut microbial community structure as determined by phylogenetic relatedness and abundance (PERMANOVA: pseudo-F= 12.69, $p< 0.001$ using weighted Unifrac distance metrics; Figure 2A). This transition was more dramatic in the first 6 months of life before beginning to stabilize at around 8 months. Ten to 12 months of age was only time period when a significant shift in microbial structure was not

observed (pseudo-F= 1.00, $p< 0.39$). Moreover, the within-group pairwise phylogenetic differences decreased over time, suggesting a convergence of microbial community structure as infants approached 1 year of age (ANOVA: F= 206.81, $p< 0.001$; Figure 2B). This more similar and stable microbiota community structure emerged after the infant was weaned from the mother and co-housed with other weanlings in social groups. As previously reported in rhesus infants [37], co-habitation in a small group facilitates the transfer of microbes leading to the adoption of a similar microbial community structure within each peer group. Temporal changes within each monkey's beta diversity were then evaluated in the 40 infants who underwent successive sample collection from 2 weeks to 12 months of age. In corroboration with our cross-sectional results, longitudinal analysis indicated that weighted Unifrac distance did decrease over the course of the first year (REML Weighted*:* $p<0.001$) with male and female infants exhibiting similar rates of phylogenetic transition ($p=0.21$; Figure 2C). Alpha diversity metrics were also dynamic and increased exponentially over time in the developing infants (Faith's Phylogenetic Diversity (FPD): $p<0.001$; Observed OTUs: $p<0.001$; and Evenness: $p<0.001$, respectively; Figure 2D). The impact of time on phylogenetic richness was also still evident in a repeated measures analysis of the 40 infants from whom it was possible to acquire samples consistently from 2 weeks to 1 year of age (REML FPD: $p<0.001$). There was no sex difference in the rate at which the microbiome diversified among these infants.

Relative phylum and genus-level abundance profiles were then evaluated at each time point using the non-parametric Kruskal-Wallis analysis of variance and the extent of these differences was substantiated using LDA effect sizes calculated through LEfSe (LDA >2, $p <0.05$) (Figure 3B). Like adult female rhesus monkeys, infant microbial profiles were dominated by

Firmicutes at all time points, accounting for 49.5% of the total reads, followed by Bacteroidetes (22%), Proteobacteria (10.1%), and Actinobacteria (7.6%) (Figure 3A). However, unlike the overall stability seen within the microbiome of mothers, the ratio of Firmicutes to Bacteroidetes increased dramatically after weaning (FDR $p<0.001$), largely due to increased colonization of genera within Firmicutes at 8 months of age (*Lactobacillus*: 6.8% at 6 months vs. 12.4% at 8 months; *Streptococcus*: 1.7% vs. 9.3%; *Ruminococcus*: 1.8% vs. 2.5%, respectively). With the exception of birth, and in accord with the composition of maternal gut, the predominant genus isolated from infant swabs was *Prevotella* (within Bacteroidetes). *Prevotella* abundances increased steadily over the first 6 months to 26.2%, surpassing levels seen in mothers before declining to 22% at one year of age.

At birth, the infant microbiome was instead characterized by elevated abundances of the genus *Bacteroides,* but these levels had diminished to <1% by 2 months of age. Facultative anaerobic *Enteroccoccus* and *Staphylococcus*, a genus commonly found on skin, were also uniquely detected in samples taken around the time of birth but these levels also diminished quickly by 2 weeks (FDR $p<0.001$ and $p<0.001$). Unlike adult females, infants were more heavily colonized by the genus *Bifidobacterium* within the phylum Actinobacteria. *Bifidobacterium* abundances peaked at 19.6% at the height of lactation (1-2 months postnatal) before diminishing to 0.06% when infants were weaned from their mothers and placed in a social group of peers (FDR $p<0.001$). *Flexispira*, a genus within Proteobacteria, mirrored this higher abundance during peak nursing, but unlike *Bifidobacterium,* levels persisted following a decrease at weaning (FDR $p=0.002$). There were no consistent sex differences in the relative abundance of common microbes in infants across the first year of life.

The timing of these transitions appeared to have a persistent influence on bacterial community structure, and deviations in bacterial composition during early infancy were associated with alterations in the rate at which a more adult-like microbiome was acquired. In keeping with studies of the human infant microbial succession, the abundance of Proteobacterial and Actinobacterial OTUs was negatively correlated with the abundance of Firmicute genes [38]. While enrichment in Actinobacteria and Proteobacteria at 2 weeks corresponded to a faster acquisition of phylogenetic diversity and a more even microbiome structure over the first 6 months, elevated levels of Spirochaetes and Firmicutes were predictive of less change in community complexity. At 2 weeks of age, a higher abundance of genera within Firmicutes, including *Faecalibacterium*, *Coprococcus*, and *Ruminococcus*, predicted a more infantile and less even community structure at 6 months of age (r=.54, p=0.008; r=-.45, p=0.033; and r=-.66, p=0.001, respectively).

Maturation of the Infant Gut. The analysis then shifted to determining when in early life the infant microbiome begins to resemble that of an adult monkey and whether the abundance of several of the more common taxa was heritable. This characterization was done through distance comparisons, which evaluated changes in the phylogenetic relatedness and abundance of the microbial profile between infants and their mothers using samples acquired from 2 weeks to 6 months of age. While the microbial community composition of infants remained distinct from that of their mothers at 6 months postnatal (weighted UniFrac; PERMANOVA: pseudo-F= 5.51, p=0.001; Figure 4A), distances between paired mother-infant samples diminished with increasing infant age and more overlap between the infant and maternal

clusters became apparent at 4 months of age. The Jaccard distance index, which converts frequencies to binary values in order to evaluate the presence/absence of taxa and does not take relative abundance into consideration, was then used to track change in the proportion of lineages shared by the two communities over time. While the microbial community structure of infants again remained distinct from their mothers at 6 months of age (PERMANOVA: pseudo-F= 4.26, p=0.001), Jaccard distances between sequence variant profiles indicated that the proportion of shared features became increasingly more similar over time. Interestingly, the infant gut shared the least taxa with the maternal gut at 2 months of age, which is the peak time point of nursing and consumption of milk. This finding is likely due to the unique content of oligosaccharides in breast milk, which selectively encourage a microbial profile that is richer in Actinobacteria and Proteobacteria [21].

It was then investigated whether the composition and abundance of bacteria in the infant gut continued to become more similar to maternal microbial profiles after the infant was weaned and housed separately from the mother. Because samples taken after the 6-month timepoint were limited to infants and there were no significant differences in the microbial composition of mothers across lactation, the ordination values from weighted Unifrac distances calculated from samples collected at 2 weeks and 2, 4, and 6 months postpartum were merged to create an average representation of the maternal microbiome. As seen in Figure 4B, dissimilarity in community structure between mothers and infants continued to decrease after 8 months; however, infant microbiota structure remained distinct from that of the mother throughout the 12-month sampling period (weighted UniFrac; PERMANOVA: pseudo-F= 3.27, p=0.005). Nevertheless, the phylogenetic richness and diversity of the infant microbiome

matured significantly following weaning and did reach maternal levels by 12 months of age (Figure 2D). Adult-like levels of microbial evenness were acquired earlier, with infants displaying a balanced bacterial distribution within the gut by 4 months postpartum on average, followed by stability through 12 months. In sum, the infant microbiome continues to be markedly different from that of the mother at one year, and a longer sampling window would be required to fully document the complete maturational process prior to puberty.

In addition to characterizing the timeline of microbiome maturation in the gut, we sought to explore the vertical transmission of gut bacteria by examining the potential heritable aspects of microbial community structure and the degree to which the abundance of specific genera in the mother would corresponded to the levels seen in her offspring. Despite evidence that the infant adopts a microbial profile more similar to that of the mother with increasing age, pairwise comparisons of the mean dissimilarity distances among microbiota profiles indicated that the gut community structure of mother-infant pairs at 6 and 12 months of infant age were not more similar to each other than they were to the profiles of infants and unrelated adult females (weighted Unifrac; Mann-Whitney *U* at 6 mos.: p=0.002 and 12 mos.: p=0.007; Figure 5). Furthermore, at both time points, sample-to-sample distances indicated that the infant microbiome was most structurally similar to other infants of the same age. Additionally, while the infant microbiomes did reach maternal levels of richness and diversity, there was not a pair-wise correspondence of alpha diversity indices within mother-infant dyads at any age point. Together, these findings suggest that macaque microbiomes are not uniquely inherited at the time of birth or through exposure to specific sources of maternal fecal microbes. Other

components of the infant environment, early diet, and later social interactions may be additional factors that have a major contributory influence on gut microbial profiles.

In keeping with these findings, there was limited correspondence in the more abundant taxa (>1%) between mothers and infants. To allow for mother-infant comparisons after 6 months of infant age, maternal taxa abundances were averaged to create a single representative value. At the level of phylum, abundances of Firmicutes and Proteobacteria in infants corresponded to maternal levels only at 12 months of age (r=.53, p=.017; r=.57, p=.01; Table 1). Levels of *Prevotella*, the most abundant genera, within the infant gut paralleled those of the mother at the time of birth (r=.94; p=.001) and the associations approached statistical significance at 6 months (r=.37, p=.054) but not at any other time point.

A greater degree of concordance was seen among the less abundant taxa comprising ≥0.01% of the microbiome (Table 1). Within infants, the abundances of Firmicutes *SMB53* and *Phascolarctobacterium* were stable over development and found to correspond with maternal levels during both the nursing period and after the infant was weaned and housed separately from the mother. *Phascolarctobacterium* is a substantial acetate/propionate-producer, and used in probiotic supplements, suggesting that some degree of protection and gut health may be conferred to the infant through vertical transfer of this member of the maternal microbiota [39].

Within the phylum Proteobacteria, the presence of *Campylobacter* within the mother was largely predictive of the levels found in the infant across the first 10 months of life, however abundances observed within the infant gut were higher, peaking at 2% at 2 months of age as compared to an abundance of only .09% in mothers (Table 1). Because none of the

rhesus infants displayed diarrheic symptoms indicative of Campylobacteriosis, the *Campylobacter* spp. present are likely non-pathogenic [40]. The potential pathogenic activity of *Campylobacter* may have also been suppressed due to exposure to fucosylated sugars in breast milk, which can inhibit bacterial adherence and penetration into the intestinal mucosa [41].

Multiple lineages of the genus *Clostridium* (from families *Lachnospiraceae, Ruminococcaceae,* and *Clostridiaceae*) were also correlated between mother and infant (Table 1). Following weaning, and infants were more likely to host *Clostridium* if the taxa was also present in their mothers and relative abundances of the genus were stable within the infant gut throughout 1 year of age. These findings concur with twin studies in humans reporting that the genera *Clostridium* are primarily inherited and are largely stable over time [2]. This correspondence between abundances found in the maternal gut and abundance in the post-weaning infant may arise only after cessation of nursing because, in a manner analogous to *Campylobacter*, breast milk has been found to inhibit the growth of certain pathogenic *Clostridium* strains, including *Clostridium difficile* [42]. Finally, to further examine the extent to which bacterial transmission from mother to offspring may have an enduring influence on the bacterial composition of the infant gut, the frequency of overlap in bacterial species was examined between mothers and their offspring when infants were 12 months of age. Of the 74 taxa that were identified at the species-level, on average 81% of maternal species appeared to be shared with infants, suggesting an influence of additional environmental factors beyond the original derivation of these bacteria from the mother.

Functional Pathway Potential. PICRUSt was utilized to identify the potential metabolic functionality of the microbiome over the first year of life. It revealed that the dynamic nature and increasing richness of the microbial composition seen in the developing gut was accompanied by progressive changes in the predicted metagenome. A comparison of the predicted KEGG Orthogs using the Jaccard dissimilarity index, a nonphylogenetic beta diversity measure, demonstrated significant clustering at different age points and suggested that the fraction of shared OTUs between samples increased over time (PERMANOVA: p=0.001). These fluctuations in the predicted pathway functionality were especially evident when evaluating the relative abundance of metabolic modules. In addition, despite evidence that microbial taxonomic composition had not yet reached maturation by 1 year of age, the levels of KEGG pathway activation were found to consistently approach those seen in the mothers, with the majority converging by the time the infant was 12 months of age (Figure 6A).

Of the 39 Level-2 KEGG Orthology groups that were identified and represented in the infant microbiome, Kruskal-Wallis testing revealed that 29 varied significantly across the first year of life. Among these categories, the functional abundance of Amino Acid Metabolism (FDR p=3.55e-35), Cell Transport and Catabolism (FDR p=2.59e-21), Signaling Molecules and Interaction (FDR p=1.64e-19), and Metabolic Diseases (FDR p=3.53e-10) were significantly elevated at 2 weeks after birth, before declining with age, whereas pathways associated with Transcription and Translation Proteins (FDR p=3.47e-4 and p=2.23e-4) had increased significantly by 12 months of age. Genes associated with the metabolism of energy and terpenoids and polyketides instead increased dramatically throughout 6 months of age before dropping and sustaining maternal levels at 8 months (FDR p=6.36e-07 and p<0.001,

respectively). Additionally, several of the identified pathways indicate a relationship with the consumption of breast milk; glycan biosynthesis and metabolism and the metabolism of cofactors and vitamins was highest during the nursing period before dropping after weaning to the levels see in the mothers. This shift directly contrasted with the pattern seen for predictions of carbohydrate metabolism, which suggested a sustained increase in pathway activation after weaning from the mother and a diet consisting exclusively of solid foods.

Despite these changes in relative abundance over time, and thus trends for effects on metabolic activity within the gut, it should be noted that the scale of these differences was small in comparison to the changes seen in the abundances of different bacterial taxa. For instance, functional gene annotations associated with metabolism were consistently inferred to be the most abundant pathway in both mothers and infants, accounting for >47% of KEGG Orthlogs, but the range of variation across all time points was only 0.8%. This conclusion is also reflected in the overall consistency of the Shannon Index within individuals over time, which reflected the evenness and abundance of bacteria within the fecal specimens (p=0.102; Figure 6B).

Microbial Trajectory & Growth Outcomes. In order to substantiate the importance of these compositional shifts within the gut microbiome, and the predicted changes in metagenomic functioning that would accompany them, the potential influence on infant growth and body size was examined. A major digestive function of gut bacteria is to enhance the extraction of energy and other nutrients from food sources and to facilitate energy storage and host metabolism. Therefore, even among normally developing and healthy infants, it may be possible that variation in the maturational succession of the gut microbiome corresponds with

differences in the rate at which infant monkeys were growing. To test this hypothesis, infant growth was examined by determining weight gain trajectories and skeletal growth was assessed by the rate of change in the length from the top of the head to the rump (crown-rump), a measure of height, from birth to 12 months of age. Growth trajectories were not different between female and male infants although there was a trend for males to be heavier at 12 months of age (p=.071). Growth variables were then evaluated in relation to microbiome maturity, defined as the degree of richness and evenness of the microbial composition, and to bacterial taxa previously identified to have time-dependent changes in abundance.

When examining the maturity of gut composition, it was found that from 2 through 6 months of age increased phylogenetic diversity and richness within the infant gut was predictive of faster weight gain (Table 2). Microbiome evenness at 2 months of age was additionally associated with an accelerated trajectory of skeletal growth. These findings are consistent with studies reporting that undernourished children have gut microbial profiles that are less mature and less diverse than those of healthy controls, and that germ-free mice colonized with immature microbiomes acquired from undernourished children grew poorly, with less muscle mass and lower bone density [43].

When evaluating individual taxa, it is also apparent that the infant growth trajectories were sensitive to the timing of enrichment of certain bacteria. Taxa within the phyla Bacteroidetes appeared to have an early influence on the trajectory of growth. At 2 weeks of age, a microbiome enriched in *Bacteroides* was associated with a faster growth rate (r=.54, p=.013), but this effect ceased to be apparent by 2 months of age, at which time *Bacteroides* levels had diminished to <1%. Significantly, members of the *Bacteroides* genus harbor extensive

polysaccharide utilization loci, which function to import and metabolize dietary and host glycans [44]. These adaptations allow *Bacteroides* to be effective consumers of the oligosaccharides in breast milk, and in doing so produce short chain fatty acids which have an important role in host metabolism. In this manner, the effect of *Bacteroides* abundance on infant growth appeared to be linked to the findings from KEGG predictions for a potential influence on the glycan biosynthesis and metabolism pathway activation, which was independently predictive of weight gain (r=.44; p=.003, Supplemental Figure 2).

Beyond 2 weeks of age, growth trajectories appear to reflect opposing influences of Firmicutes and Proteobacteria. As earlier reported, levels of Firmicutes were inversely related to Proteobacteria abundance, and Firmicutes acquisition led to greater microbial diversity within the gut and appeared to be a signature of a more mature microbiome community [45]. Infants colonized with elevated abundances of Firmicutes at 2-6 months of age displayed faster growth trajectories, while microbiomes consisting of over 30% Proteobacteria at 2 and 4 months were characterized by slower weight gain (Table 2). Among human infants, reduced bacterial diversity and sustained increases in Proteobacteria have been proposed to be a sensitive indicator of dysbiosis and disease risk, including for the development of necrotizing enterocolitis [46,47]. Proteobacteria did not reach pathological levels in these healthy rhesus monkey infants, but Proteobacteria may still compete with taxa conducive to growth, such as Firmicutes and Bacteroidetes.

Firmicutes are efficient harvesters of energy and are involved in short chain fatty acid production. In adults, their overabundance has been linked to symptoms of metabolic disorder [48], but in infants, metabolic efficiency is conducive to rapid growth. When looking within the

Firmicutes phylum, the 4-month abundance of taxa within the Clostridia class, as well as the genus *Lactobacillus*, appeared to be predictive of the monkeys' weight gain trajectories. Alternatively, the abundance of the *Lactobacillus*, *Blautia*, and *Coprococcus* genera at 2 months of age corresponded to skeletal growth. Infant growth trajectories also corresponded to the upregulation of components of several pathways involved in central energy and carbohydrate metabolism, including those involved in glycolysis/gluconeogenesis, pyruvate metabolism, galactose metabolism, purine and pyrimidine metabolism, and fructose and mannose metabolism, all of which are functions attributed to Firmicutes.

DISCUSSION

Description of the microbial succession within the infant gut

The development of the gut microbiome in young infants is a critical factor associated with long-term gut health and overall well-being. This study investigated the early acquisition and progression of the gut microbiome of the infant rhesus macaque, and how the process is associated with bacterial taxa originating from the mother. The findings described in this chapter represent one of the longest, natural evaluations of the gut microbiome in infant rhesus monkeys. Consistent with studies in humans, the bacterial composition in the maternal gut is observed to be stable across the 6-month lactation phase, whereas the infant gut is highly dynamic, and the maturation of its microbiome community structure was found to be a nonrandom process [36,46]. The progressive nature of infant gut microbial colonization was apparent through alterations in the overall community composition, as well as increases in richness, diversity, and evenness over the first year of life [17,49]. Inter-animal dissimilarity

decreased over time, highlighting the convergence of a more complex and less heterogeneous community. These compositional shifts within the gut community structure were also indicative of changes in the predicted metagenomic activity of the microbiota and were associated with infant growth. This suggests that anomalous sequential patterns of microbial succession during the first year of life may be a predictive biomarker of poorer developmental health outcomes.

Our findings indicate that there may be several critical windows of development during the first year of life with distinctive microbial signatures. Following birth, the infant microbiome is characterized by low diversity due to the predominance of taxa within Actinobacteria and Proteobacteria. Following the period of peak nursing between 1 and 2 months of infant age, the gut microbial composition diversifies, and Firmicutes, and to a lesser extent Bacteroidetes, become more dominant. At the time of weaning and the transition to exclusive consumption of solid foods, infants rapidly acquire a more adult-like microbial community structure, similar to that seen in human infants with the cessation of nursing [50]. Taxa associated with breastfeeding were largely diminished, with Actinobacteria decreasing to >1% abundance, and the ratio of Firmicutes to Bacteroidetes became more similar to that of mothers. Despite this adult-like gut microbial structure and maturation of the predicted functional capacity of the microbiota, compositional differences remained in the gut microbiome between the 1-year-old infants and their mothers. This difference indicates that a longer period of maturation is required to fully see the progression of bacterial succession from infant to an adult-like state.

The strength of the effect of age on the taxonomic succession and its overall consistency across infants suggests that the timing of these transitions are of developmental importance. For instance, many of the initial bacteria in the young infant's gut are facultative anaerobes,

including the Proteobacterium *Enterococcus* and the Firmicute *Staphylococcus.* These taxa have been found to facilitate the transition of the gut microbiota from an aerobic environment to an anaerobic environment, enabling the shift to obligate anaerobes with increasing abundance of *Bifidobacterium* and *Prevotella* [51]. Alterations in these patterns, especially the enduring enrichment of Proteobacteria beyond the first postnatal weeks, or premature colonization by genera within Firmicutes, such as *Faecalibacterium* and *Coprococcus*, have been associated with reductions in microbiome richness and delayed maturation [45–47]. If more extreme, these types of delays in microbial richness and diversity may be indicators of an infant that will progress to a failure to thrive. Although this was a study of otherwise healthy infants, this was evidenced by our finding that an immature microbiome was predictive of a slower rate of physical growth, both in body weight and skeletal height.

Furthermore, these microbial trajectories were consistent across females and males, despite known sex differences in the hormones, behavior, and nutritional requirements of neonates [52], as well as past findings documenting sex differences in gut microbial communities between male and female macaque adults [23]. Because infancy is characterized by less sex-specific differences and is a time of relative quiescence for the reproductive gonadal axis [53], it is possible that the emergence of sex differences in the gut becomes more apparent after puberty, as has been reported in studies of opposite-sex twins [3,54]. Future studies that monitor the gut microbiome of the rhesus monkey beyond the first year of life are needed to both determine the age at which the gut completely acquires an adult-like microbial profile and also to establish when sex differences in microbial structure emerge.

Maternal contributions and infant diet shift the infant gut microbiome

Following the initial microbial inoculation at the time of vaginal delivery, microbial seeding of the infant gut continues during the postnatal period and is reinforced through breastfeeding and maternal contact. However, the transmission of genera from mother to infant does not indicate whether vertically acquired microbes are transient occupiers or are actively colonizing the infant gut. Thus, an enduring maternal influence on the infant gut was evaluated through taxa that persisted after infants were weaned from their mothers and relocated to peer-housing. Within our study, weaned infants exhibited a large proportion of shared bacterial species with their mothers, but there was little correspondence between infants and their mothers with regard to community structure and the abundances of prevalent taxa.

A maternal influence on the microbial succession of the infant gut was more apparent through the dramatic compositional shift seen in the infant microbiome following weaning from the mother and the cessation of breastfeeding [55]. During lactation, the gut microbiome of infants was characterized by a higher rate of microbial diversification and colonization by oligosaccharide-consuming commensal taxa, and relocation away from the mother into peer-housing and the exclusive consumption of solid foods resulted in the rapid adoption of a more stable and adult-like microbiome, as marked by increases in taxa within the phylum Firmicutes. These shifts in composition were accompanied by changes in the functional repertoire of the infant microbiome. PICRUSt analysis revealed that the predicted metagenomes related to glycan biosynthesis and metabolism pathways were elevated throughout lactation, before plummeting to maternal levels at 8 months of age. These KEGG pathways aid in the digestion of

glycans extracted from oligosaccharides and other carbohydrates, thereby facilitating lactose utilization in breast milk and contributing to host metabolism [44,56,57]. Consistently, the predicted activation of glycan biosynthesis pathways at 2 weeks of infants age, a period of exclusive breastmilk consumption, was associated with infant growth trajectories.

Taxonomic dissimilarity to the microbial composition of human infants

Despite similarities in the overall patterns of microbial succession between human and rhesus infants, there are several notable differences in the gut microbial colonization between primate species. The first is the commensal *Lactobacillus*, which displays a striking increase in breastfed human infants over the first 6 weeks of life (potentially reaching 70% of the gut microbial composition), but does not exhibit substantial increases in the gut of the rhesus infant until post-weaning and at much lower abundances (>10%) [58]. This discrepancy between species may have several origins. While *Lactobacillus* dominates the conserved human vaginal microbiota, the vaginal microbial composition of non-human primates is highly diverse, and *Lactobacilli* abundance is more sparse, suggesting less transmission of the taxa through vaginal delivery [24,59]. Furthermore, the *Lactobacillus* spp. that are present in rhesus monkeys also differ from those found in human milk and infants, and are not efficient consumers of the oligosaccharides found in breastmilk [60]. While lactic acid bacteria have also been identified in the milk of rhesus monkey (*L. gasseri*, *L. rhamnosus,* and *L. plantarum*) [61], these taxa differed from those colonizing infants (primarily *L. salivaris*, *L. ruminis*, and *L. pontis*). Taken together, this suggests that while vaginal seeding and breast milk constitute major sources of *Lactobacilli* to the human infant, rhesus infants are colonized by other means.

The succession of taxa within Bacteroidetes also differed between rhesus monkey infants and human infants born in Western countries [62]. *Bacteroides*, for instance, is largely abundant from birth until weaning in human infants, whereas rhesus infants only show enriched abundance from birth to 2 weeks postnatal. This is potentially related to differences in the maternal diet; *Bacteroides* are typically associated with the consumption of a high-fat or protein-rich diet and are abundant within the gut microbiota of adult humans from Western countries [62,63]. Conversely, the preponderance of *Prevotella* strains within rhesus monkeys has been linked with enhanced potential for carbohydrate catabolism and corresponds to their consumption of a fiber-rich diet [40]. This is evidenced within our sample, as *Bacteroides* failed to reach 1% in rhesus mothers. Because *Bacteroides* were rare in the gut microbiome of adult female rhesus, and lower levels are seen in infants born through Caesarean delivery [55,64], it was hypothesized that this early and transient colonizer primarily derives from the mother through vaginal seeding and its growth is largely suppressed by the introduction to solid foods.

In contrast to the prominent colonization of *Bacteroides* in the gut microbiota of humans from Western countries [62], the surveyed macaque gut community exhibited a predominance of *Prevotella*. Initially, it was hypothesized that abundances of *Prevotella* within rhesus infants would correspond with those of their mothers during the 6 months of cohabitation. However, further research revealed that members of the Bacteroidetes phylum within the gut are generally environmentally determined, and high levels of Prevotella are seen in response to diets enriched in plant carbohydrates, such as the one provided by commercial monkey biscuits [65,66]. *Prevotella* have also been previously identified as an abundant colonizer in the reproductive tract of the rhesus [67] and may be transmitted during vaginal

delivery. Consistently, infant levels of *Prevotella* corresponded with those of their mothers within several days after birth and again at 6 months of age, when their diets are transitioning to biscuits and they are still housed in mother-infant dyads, and therefore, continuously exposed to maternal microbes. Social interactions and maternal and infant diet, in addition to microbial seeding, should consequently be considered when evaluating which transmitted strains are more likely to adapt to and persist in the infant gut.

Our findings further characterize the process through which the infant acquires microbial taxa and develops a more adult-like gastrointestinal microbiome. However, the phenomenon of vertical transmission is complex and may result from the interplay of microbial inoculation during delivery, shared environmental conditions, and common genetically encoded factors, including those that influence gut and vaginal physiology, among other variables [2,68–70]. Further research is needed to definitively determine the degree to which each of these factors uniquely account for the transmission of microbes from mother to infant. In order to better differentiate between vertically and horizontally transmitted bacterial lineages in the rhesus gut microbiota, it will be important that future studies utilize strain-specific technology, such as next-generation sequencing, since bacteria exhibit considerable strain heterogeneity [71]. Meta-transcriptomics are also needed to study the in vivo gene expression to determine whether these vertically transmitted strains are transcriptionally active [72]. Future studies that demonstrate the interrelations between the vaginal, skin, gastrointestinal, milk, and environmental microbiomes of the mother and infant are also warranted to determine the origins and transmission of bacterial strains. It should, however, be noted that the majority of enduring bacterial strains shared with the infant likely originate in the maternal gut [14].

In summary, the current study investigated the early acquisition and development of the infant gut microbiome and the association of the microbial succession with infant developmental outcomes, including weight gain and skeletal growth. Elucidating the nuances of microbial transmission and establishing a representation of normal patterns of microbial progression is essential for devising strategies to identify alterations in gut microbial composition and facilitating the establishment and maintenance of healthy gastrointestinal tract microbiota in early life. Though there are distinct differences in the taxonomic composition of the gut microbiota of the rhesus monkey as compared to reports in human infants, our findings provide further evidence that the infant rhesus macaque is an informative model for research on gut development and evaluation of the successive changes in the gut microbiota in early life. Such knowledge is important not only for furthering our understanding of non-human primate growth and development but also for advancing the utility of infant monkeys as models of human child health and development.

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Chapter 2: FIGURES & TABLES

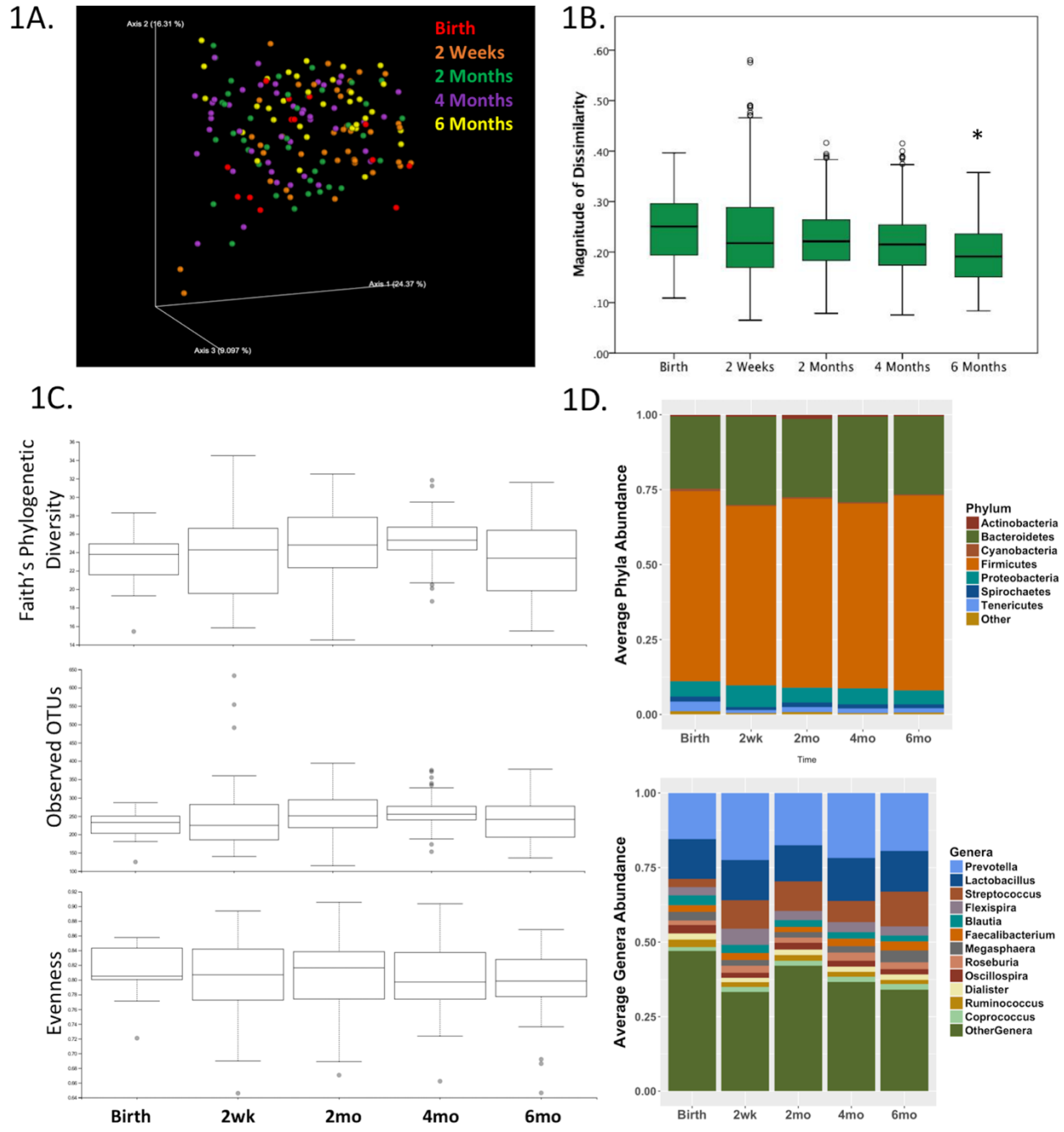
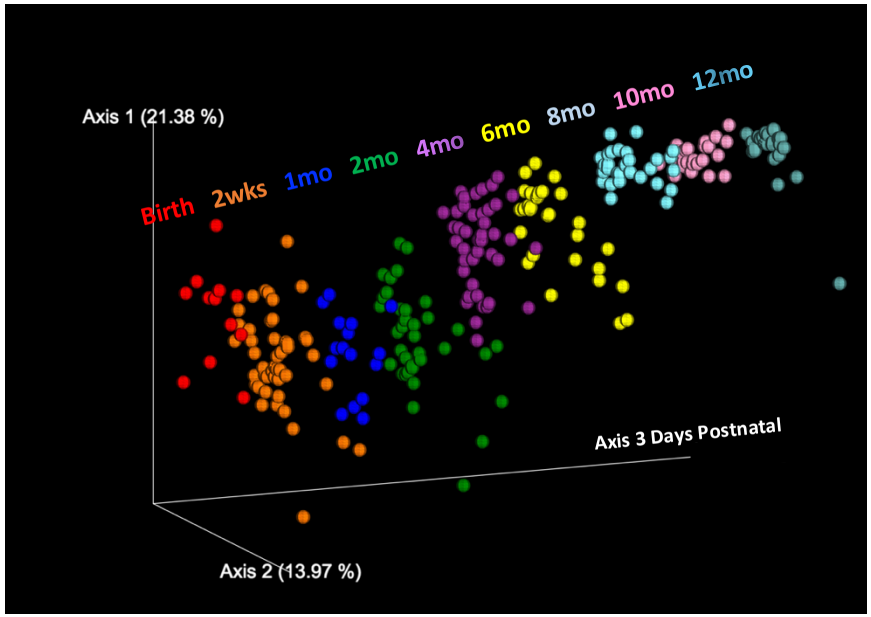


Figure 1. Maternal microbiome diversity metrices and composition over lactation. **1A.** Principle Coordinate Analysis (PCoA) by weighted Unifrac distance was used to show the overlap of maternal samples from different timepoints over the lactation period. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. **1B**. Dissimilarity distances indicated that there were small but significant decreases in inter-individual differences in microbial composition at 6 months postpartum, however the effect of time was not maintained in an LME model. **1C.** There were no differences in Faith's Phylogenetic Diversity, mean total OTUs, or sample evenness in mothers. **1D.** Relative abundance of gut bacterial taxa at the level of phylum and genus. At the phylum-level, mothers were primarily colonized by Firmicutes, however *Prevotella*, within Bacteroidetes, was the most abundant genus. Taxa constituting <1% abundance are collapsed into the 'Other' category. *p<0.05

2A.



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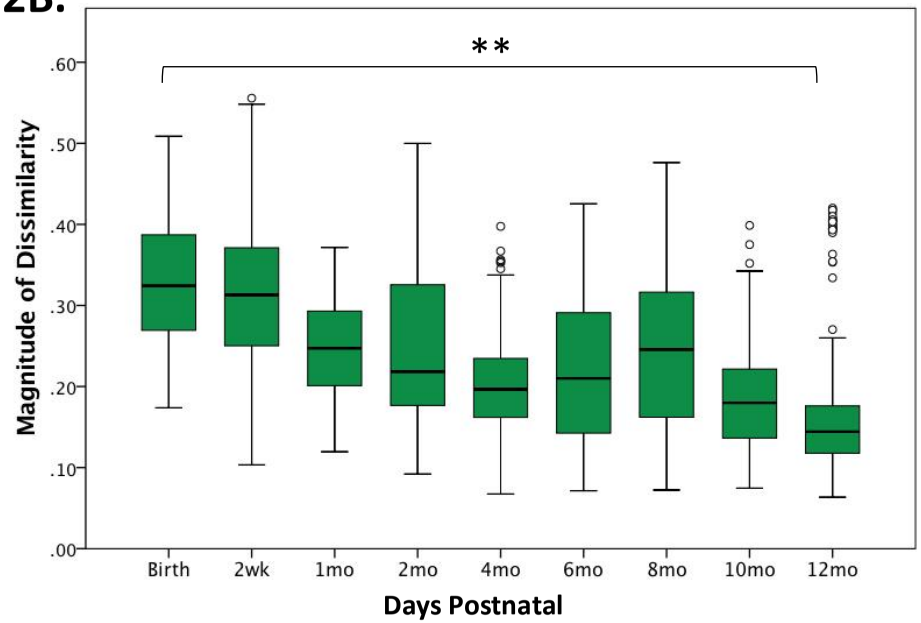

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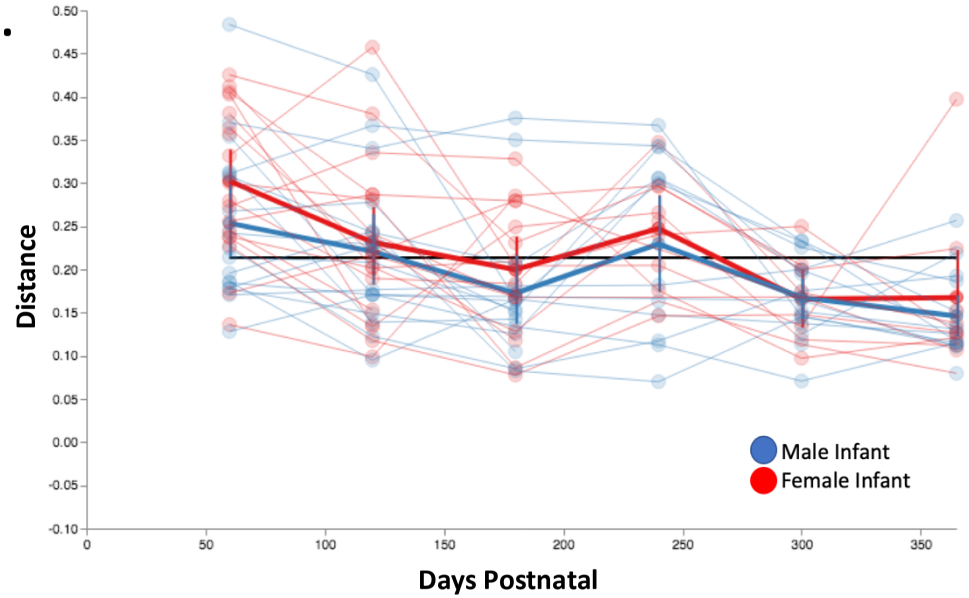


Figure 2. Infant microbial structure and composition is dynamic across the first year of life. **2A.** PCoA plot based on weighted Unifrac distance with axes for principal coordinate 1 (21.38%), principle coordinate 2 (13.97%), and infant age. **2B**. Boxplot of stability of species-level composition profiles over time as measured by weighted Unifrac dissimilarity of infants. The relative distance between samples decreased with infant age, indicating a convergence in gut microbiota communities. **2C.** Volatility plot of weighted Unifrac distances across time in infants along PCoA Axis 1. There were no sex differences in the longitudinal trajectory of phylogenetic transition. In boxplots, the horizontal line represents the median value and the box is the first and third quartiles; error bars indicate 95% confidence of median. ***p*<0.001.

2D.

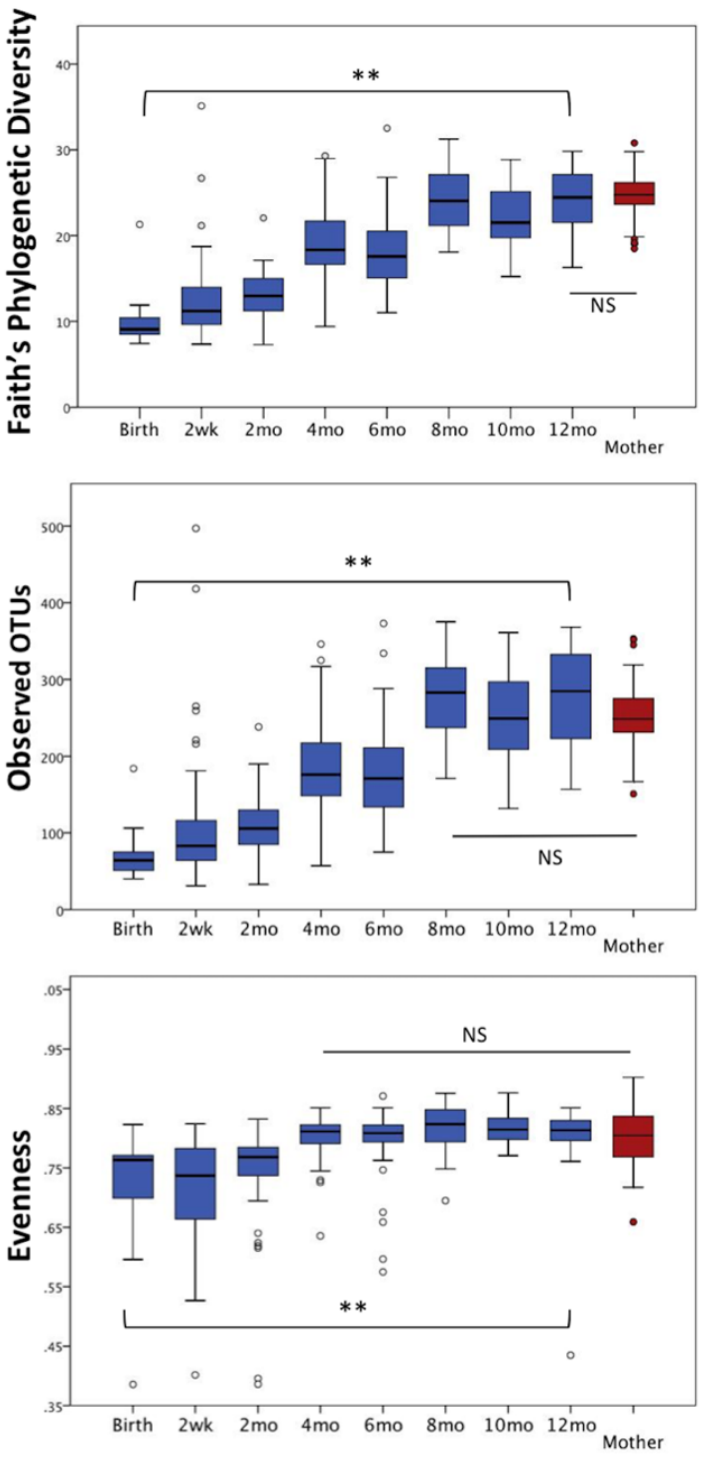


Figure 2 cont.: Infant microbial structure and composition is dynamic across the first year of life. **2D.** Alpha diversity measures of the infant microbiome across age. Faith's Phylogenetic Diversity estimations, mean total OTU count, and sample evenness are shown for each time point. Diversity metrics for the mother were the average of samples from 2 weeks and 2 through 6 months postpartum and are denoted in red. In boxplots, the horizontal line represents the median value and the box is the first and third quartiles; error bars indicate 95% confidence of median; * $p<.05$; ** $p<0.001$. NS, non-significant.

3A.

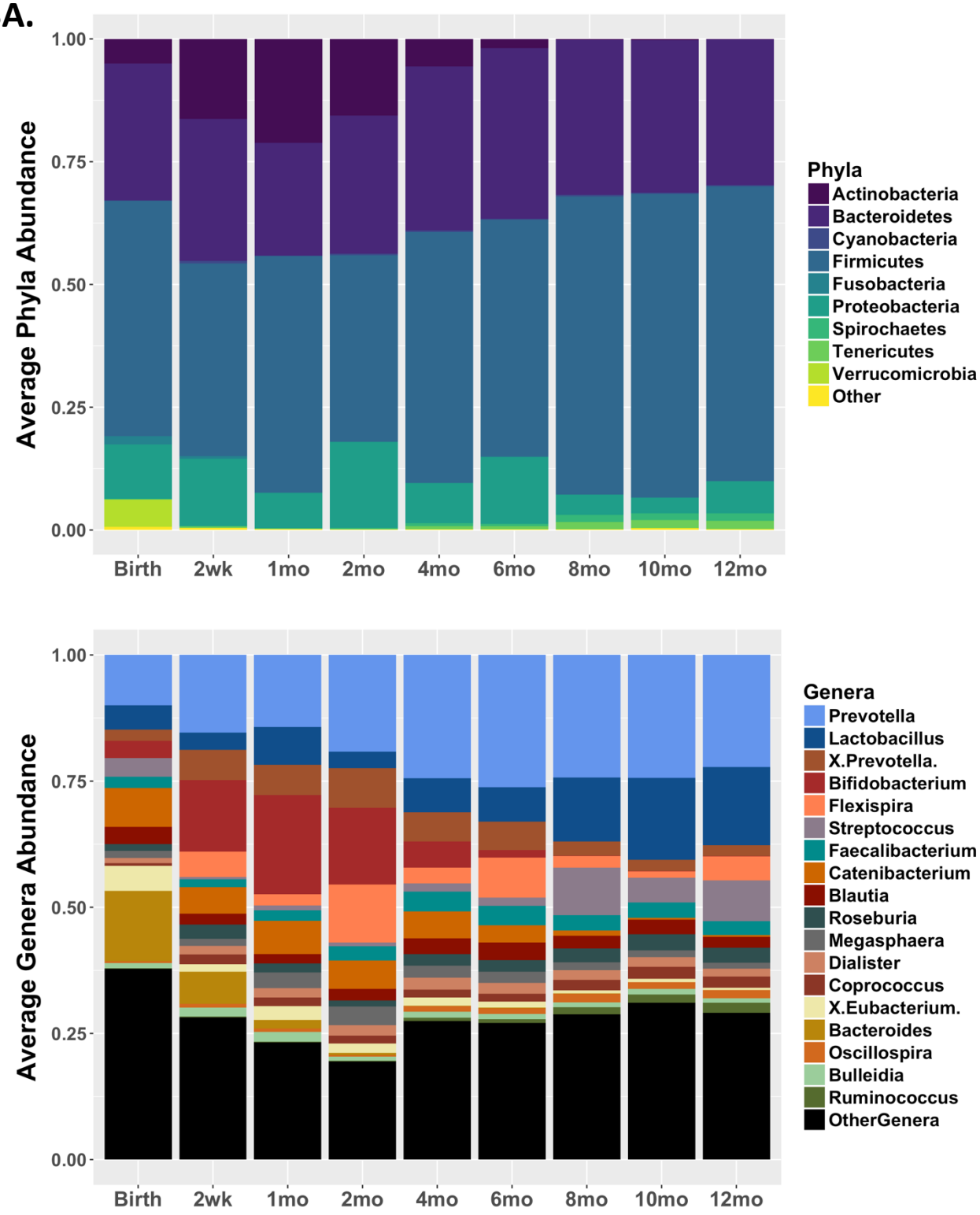


Figure 3. Infant taxonomic composition from birth to 12 months of age. **3A.** The microbial profile relative abundance at the level of phylum and genus. Taxa constituting <1% abundance are collapsed into the 'Other' category.

3B.

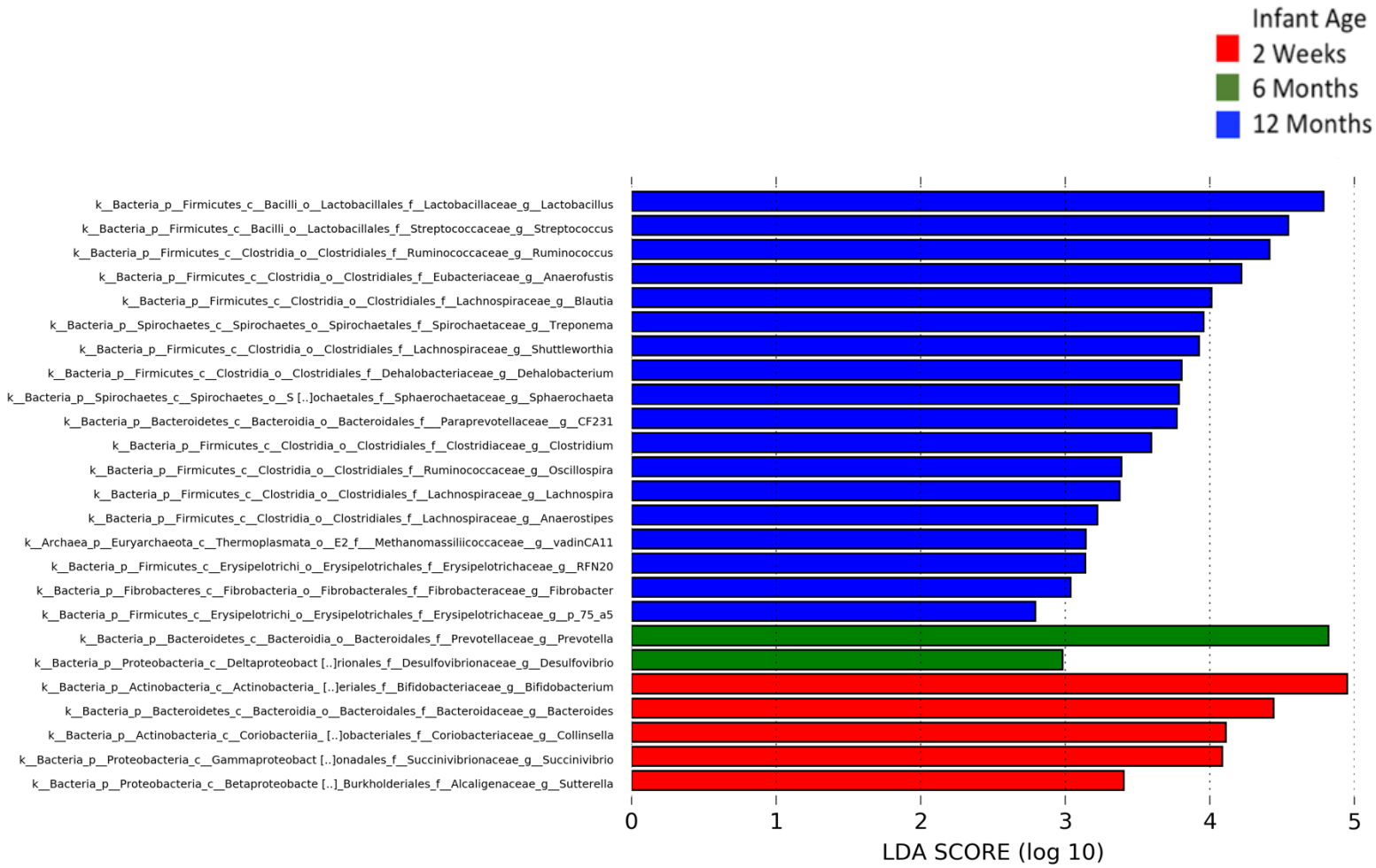


Figure 3. Infant taxonomic composition from birth to 12 months of age. **3B.** Taxa at the genus level differentially represented between major developmental timepoints: 2 weeks, 6 months, and 12 months of infant age as identified by linear discriminant analysis coupled with effect size (LEfSe). (LDA > 2, P < 0.05) Red box: enriched in Infants at 2 weeks of age; green box: enriched in infants at 6 months of age; blue box: enriched in infants at 12 months of age

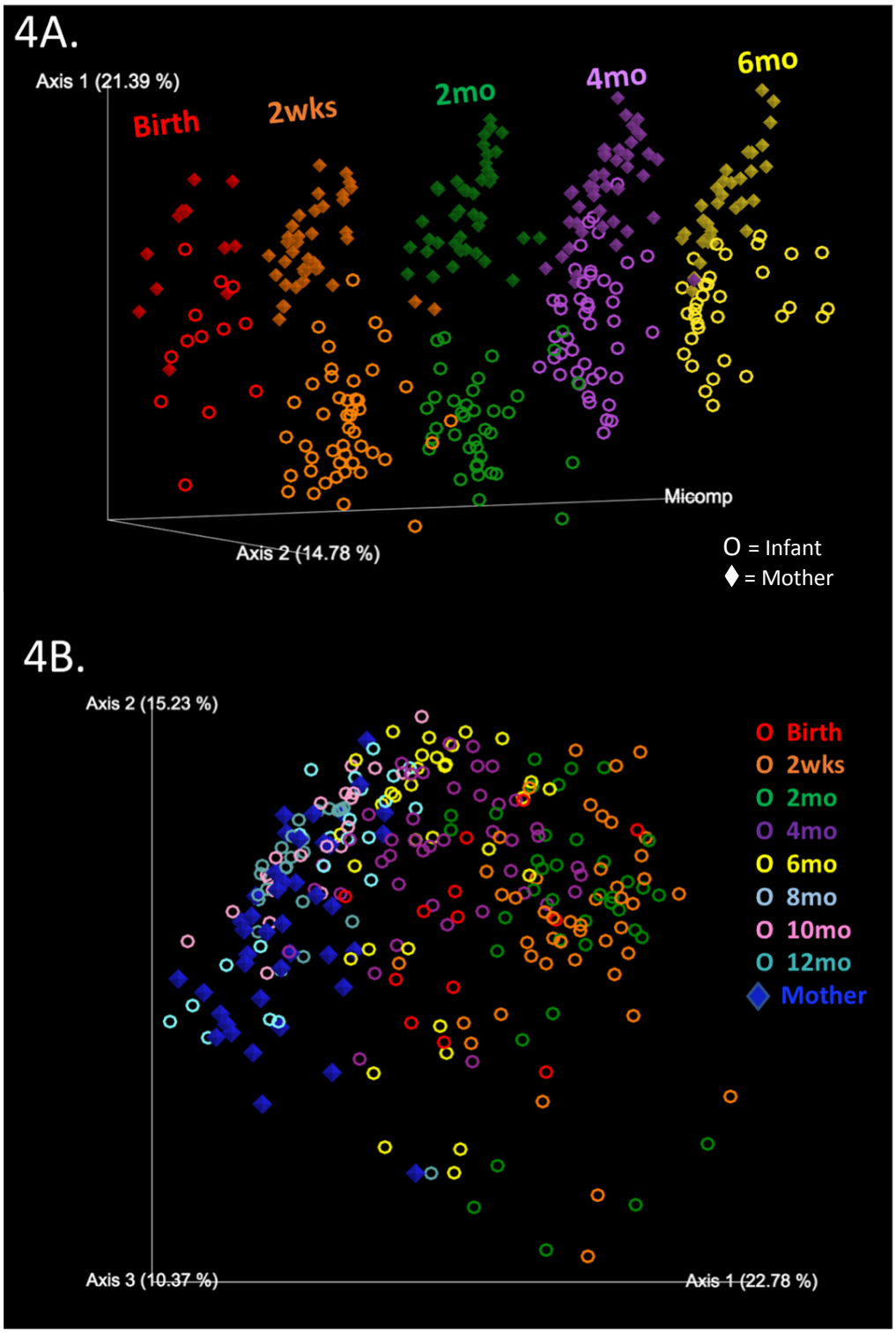


Figure 4. Maturation of the community structure of the infant microbiome through beta diversity. **4A**. Principal coordinates analysis (PCoA) derived from weighted UniFrac distances among samples from mothers and infants at birth, 2 weeks and 2, 4, and 6 months of infant age. For axis 1 and 2, in square brackets, the percent of variation explained was reported. Infant age is displayed on axis 3. **4B**. PCoA plots of bacterial beta diversity illustrating infant distances from birth until 12 months of age as compared to average maternal distances. Infants are denoted with an open circle and mothers are indicated with a filled diamond. Infant beta diversity progressively approached maternal distances but remained distinct at 12 months postpartum (p=0.007).

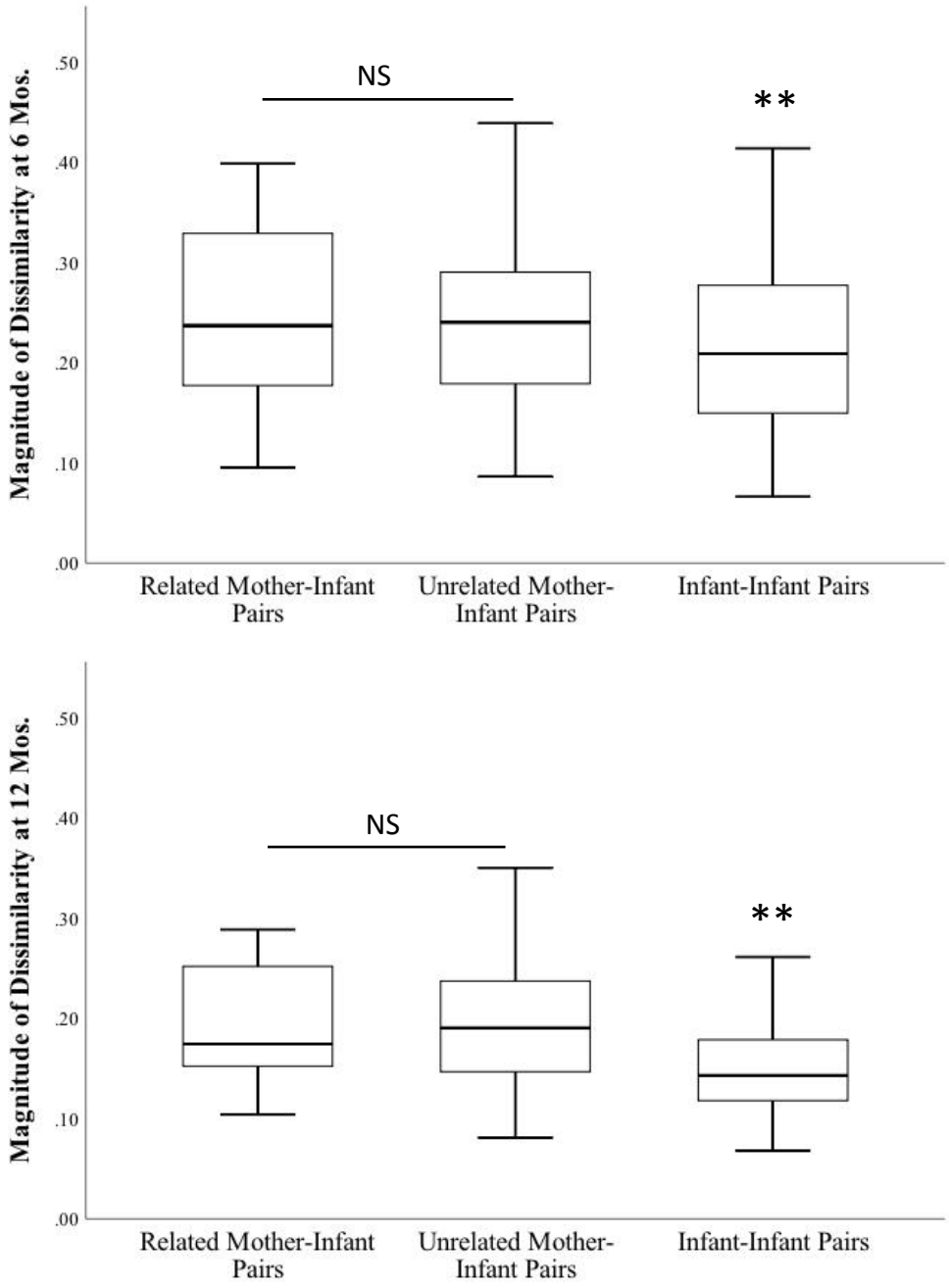


Figure 5. Analyses of inter-individual weighted Unifrac distances illustrate the magnitude of dissimilarity in the gut microbial profiles. For the distance comparison at 12 months of infant age, maternal abundances from 2 weeks and 2-6 months of age were averaged to create a composite abundance. At 6 and 12 months of infant age, there was no difference in the dissimilarity in microbiota community structure between related mother-infant pairs and unrelated pairs. Infant microbiota community structure was most similar to that of other infants. In both plots the median is given as a line, the 25th and 75th percentiles are the top and bottom of the boxes, and the error bars indicate 95% confidence of median. Statistical significance was assessed by Mann-Whitney test; **P ≤ 0.01. NS, non-significant.

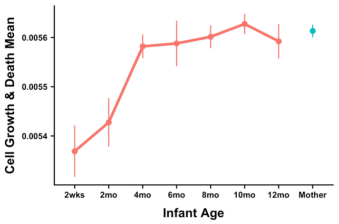
Table 1.

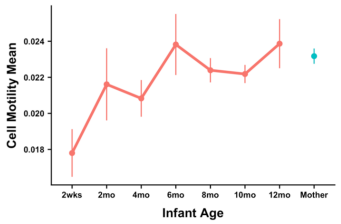
		2 Wks.	2 Mos.	4 Mos.	6 Mos.	8 Mos.	10 Mos.	12 Mos.
	Sample Size	N=36	N=37	N=41	N=31	N=17	N=18	N=19
Firmicutes	r	0.273	0.24	0.136	0.118	-0.217	-0.036	.527*
	p-value	0.151	0.218	0.483	0.559	0.437	0.891	0.02
Acidaminococcus	r	*0.659***	-0.024	*.867***	0.005	-0.134	-0.112	0.064
	p-value	*0.001*	0.904	*<.001*	0.978	0.621	0.659	0.789
Clostridium	r	ND	0.057	0.043	-0.026	*.953***	*.586**	*.542**
	p-value		0.737	0.787	0.889	*<.001*	*0.011*	*0.014*
Phascolarctobacterium	r	*.348**	0.27	*.321**	*.432**	-0.003	-0.047	*.567***
	p-value	*0.037*	0.106	*0.041*	*0.015*	0.99	0.854	*0.009*
SMB53	r	0.072	0.283	*.384**	*.426**	-0.103	*.938***	*.947***
	p-value	0.677	0.09	*0.013*	*0.017*	0.695	*<.001*	*<.001*
Proteobacterium	r	0.25	0.257	0.364	0.114	0.19	0.114	.566*
	p-value	0.192	0.188	0.052	0.57	0.497	0.663	0.011
Campylobacter	r	0.317	.481**	-0.028	.464**	.614**	.755**	-0.034
	p-value	0.059	0.003	0.86	0.009	0.009	*<.001*	0.887
Actinobacteria	r	0.118	0.27	0.196	-0.082	0.057	-0.127	0.059
	p-value	0.543	0.165	0.307	0.686	0.839	0.628	0.811
Alloscardovia	r	-0.9	-0.079	-0.148	.676**	.628**	-0.112	ND in Infant
	p-value	0.6	0.642	0.356	*<.001*	0.007	0.658	
Collinsella	r	0.035	.529**	.358*	0.2	-0.366	-0.401	0.323
	p-value	0.837	0.001	0.022	0.281	0.149	0.099	0.165
Spirochaetes	r	-0.189	0.206	.498**	.570**	0.429	.642**	0.395
	p-value	0.327	0.293	0.006	0.002	0.111	0.005	0.094

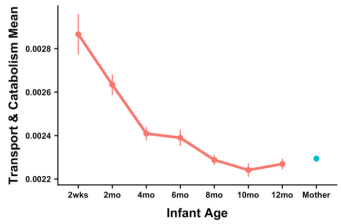
Table 1. Spearman Correlations between matched infant and maternal bacterial taxa. Comparisons at 8-10 months of infant age utilized an averaged maternal abundance created from samples collected at 2 weeks and 2-6 months postpartum. ND = Levels not detected in infant. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

6A.

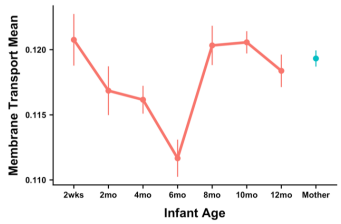
Cellular Processes

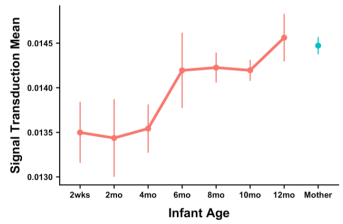




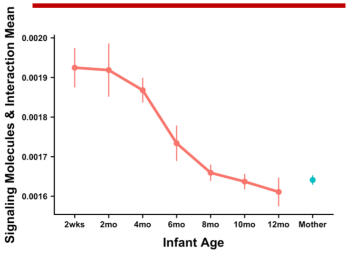


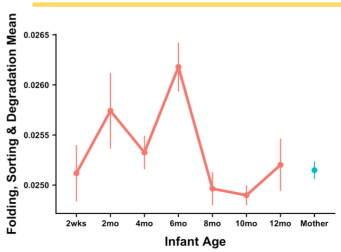
Environmental Information Processing

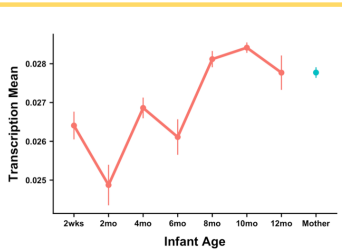




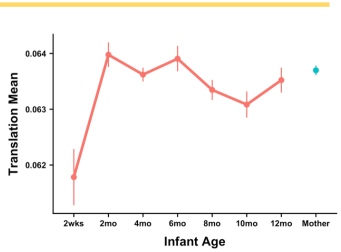
Genetic Information Processing

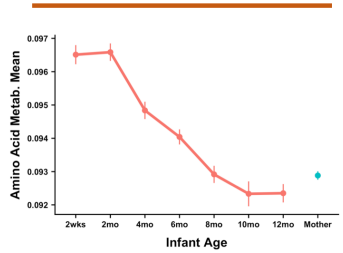


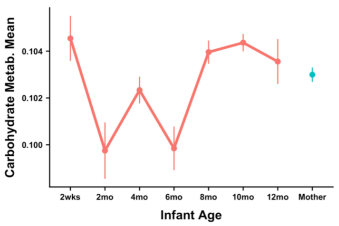


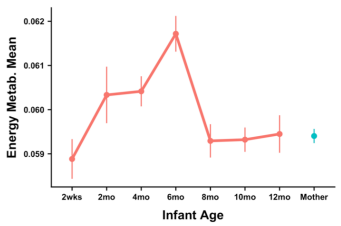


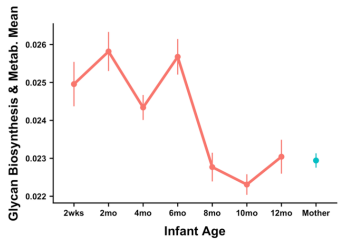
Metabolism

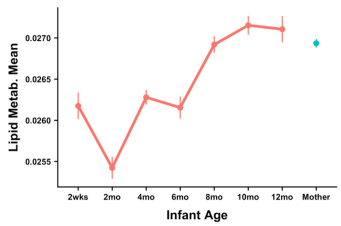


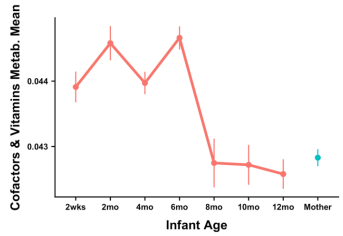




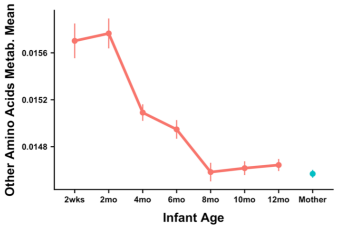


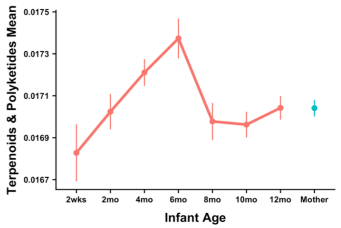


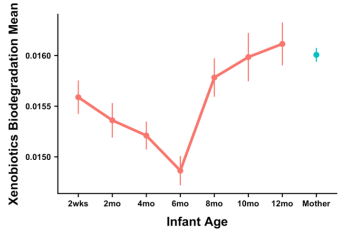


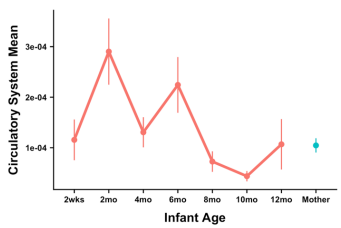


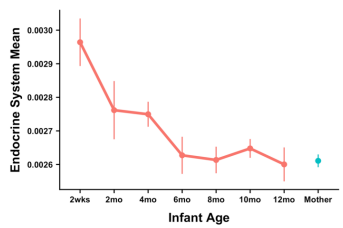
Organismal Systems



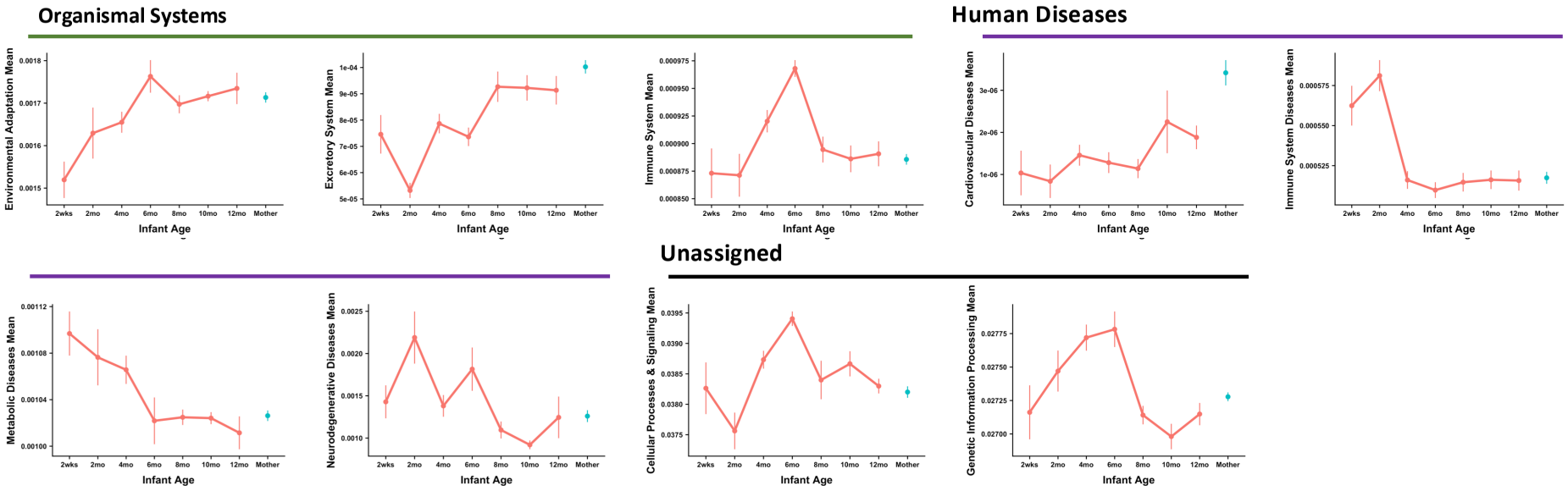








6A. Cont.



6B.

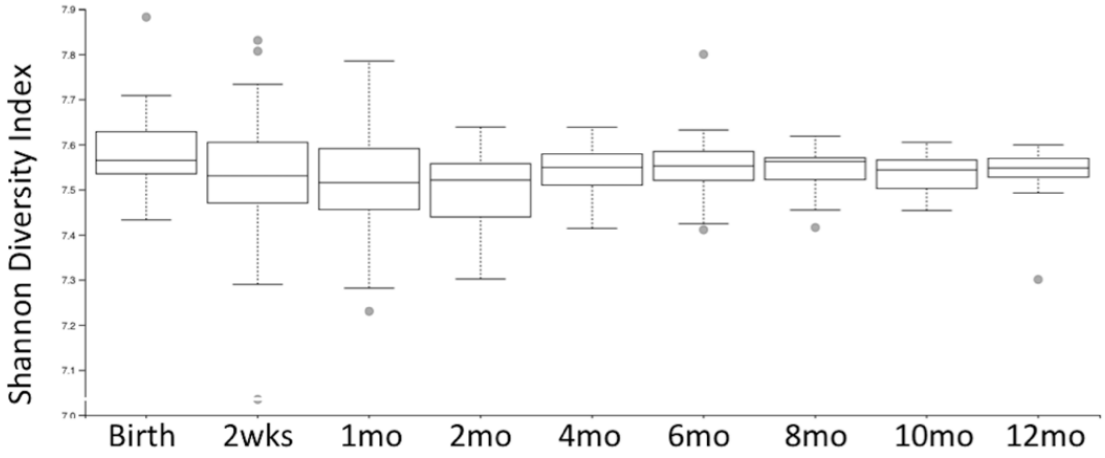


Figure 6. Predictive functional profiling of microbial communities using 16s rRNA marker genes. **6A.** Functional KEGG Ortholog Predictions at level 2 based on changes in microbiota profiles in infants at regular intervals through 12 months of age (indicated in red). KEGG values for the mother were the average of samples from 2 weeks and 2 through 6-months postpartum and are denoted in blue. Values represent means ± S.E. By 12 months, predicted activation in infants reached maternal levels for most pathways. On average, functional predictions of pathways related to metabolism stabilized following weaning from the mother and exclusive consumption of solid foods **6B.** Shannon Diversity Index of KEGG Orthologs did not vary much with infant age suggesting that sample evenness was consistent over time (*p* = .102*)*.

Table 2.

		Rate of Weight Gain				Rate of Crown-Rump Growth			
	Infant Age	2 Wk.	2 Mos.	4 Mos.	6 Mos.	2 Wk.	2 Mos.	4 Mos.	6 Mos.
Alpha Diversity									
Faith's Phylogenetic Diversity	r	-0.243	0.371	.406*	.536*	-0.02	0.277	0.116	-0.10
	p-value	0.252	0.081	0.026	0.01	0.929	0.212	0.563	0.664
Evenness	r	-0.209	0.401	.429*	0.166	0.17	.586**	0.111	0.121
	p-value	0.327	0.058	0.018	0.459	0.449	0.004	0.582	0.613
Observed OTUs	r	-0.267	.456*	.408*	.536*	0.065	0.306	0.132	-0.027
	p-value	0.207	0.029	0.025	0.01	0.773	0.166	0.511	0.908
Firmicutes	r	-0.162	0.373	.460*	.415*	0.394	.657**	0.075	-0.031
	p-value	0.45	0.079	0.011	0.049	0.07	0.001	0.717	0.893
Lactobacillus	r	-0.106	0.205	.377*	0.136	-0.033	.482*	0.064	0.109
	p-value	0.622	0.349	0.04	0.536	0.885	0.023	0.757	0.638
Faecalibacterium	r	-0.32	0.201	.392*	0.298	0.187	0.081	0.124	-0.087
	p-value	0.127	0.358	0.032	0.168	0.406	0.721	0.547	0.708
Blautia	r	0.02	0.325	.421*	0.23	0.13	.585**	-0.007	-0.337
	p-value	0.925	0.131	0.021	0.291	0.563	0.004	0.971	0.136
Coprococcus	r	-0.326	0.045	.406*	.528**	-0.192	.569**	-0.083	-0.242
	p-value	0.119	0.84	0.026	0.01	0.391	0.006	0.688	0.29
Ruminococcus	r	-0.219	.499*	0.333	0.262	-0.137	0.226	-0.171	-0.073
	p-value	0.305	0.015	0.072	0.228	0.545	0.313	0.404	0.753
Bacteroidetes	r	0.078	0.375	0.058	-0.048	-0.133	0.368	0.067	0.11
	p-value	0.716	0.078	0.76	0.828	0.557	0.092	0.739	0.636
Prevotella	r	-.498*	.496*	0.093	-0.143	-0.405	0.34	0.096	0.075
	p-value	0.013	0.016	0.625	0.514	0.061	0.122	0.632	0.748
Bacteroides	r	.541**	0.027	-0.244	0.407	0.204	0.241	-0.202	0.338
	p-value	0.006	0.901	0.193	0.054	0.363	0.28	0.312	0.133
Proteobacteria	r	0.087	-0.388	-.38*	-0.248	-0.144	-.57**	-0.107	-0.072
	p-value	0.687	0.067	0.044	0.253	0.522	0.006	0.407	0.757
Flexispira	r	-0.187	-0.408	0.006	-0.183	-0.103	-.59**	-0.027	0.002
	p-value	0.38	0.053	0.975	0.404	0.647	0.004	0.893	0.992

Table 2. Correlations between bacterial taxa during lactation period and trajectory of growth from birth to 12 months of age. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

CHAPTER 2: SUPPLEMENTAL MATERIALS

Supplemental Table 1: Ingredient composition, as well as mineral and vitamin concentrations, for the diet fed to mothers and older infants[1]

Ingredients		**Vitamins**	
Protein, %	18.05	Carotene, ppm	1.70
Carbohydrate, %	68.93	Vitamin K, ppm	3.20
Fiber (Crude), %	4.60	Thiamin, ppm	8.10
Fat (ether extract), %	13.02	Riboflavin, ppm	8.60
Minerals		Niacin, ppm	113.00
Ash, %	5.20	Pantothenic Acid, ppm	60.00
Calcium, %	0.90	Choline Chloride, pp	1200.00
Phosphorus, %	0.60	Folic Acid, ppm	7.90
Phosphorus (non-phytate), %	0.35	Pyridoxine, ppm	14.00
Potassium, %	0.73	Biotin, ppm	0.10
Magnesium, %	0.17	B12, mcg/kg	73.00
Sulfur, %	0.23	Vitamin A, IU/gm	20.00
Sodium, %	0.25	Vitamin D3 (added), IU/gm	6.70
Chloride, %	0.37	Vitamin E, IU/kg	110.00
Fluorine, ppm	20.00	Ascorbic Acid, mg/gm	0.50
Iron, ppm	230.00		
Zinc, ppm	110.00		
Manganese, ppm	94.00		
Copper, ppm	21.00		
Cobalt, ppm	0.55		
Iodine, ppm	1.30		
Chromium (added), ppm	0.01		
Selenium, ppm	0.37		

Full list of Ingredients: Ground Corn, Dehulled Soybean Meal, Wheat Middlings, Whole Wheat, Porcine Animal Fat Preserved with BHA and Citric Acid, Corn Gluten Meal, Whey, Dehydrated Alfalfa Meal, Dried Beet Pulp, Ground Soybean Hulls, Fish Meal, Sucrose, Calcium Carbonate, Dicalcium Phosphate, Brewers Dried Yeast, Salt, L-Ascorbyl-2-Polyphosphate (Stabilized Vitamin C), DL-Methionine, Menadione Dimethylpyrimidinol Bisulfite (source of Vitamin K), Pyridoxine Hydrochloride, Cholecalciferol, Folic Acid, Vitamin A Acetate, Calcium Pantothenate, Choline Chloride, DL-Alpha Tocopheryl Acetate (Form of Vitamin E), Vitamin B-12 Supplement, Nicotinic Acid, Riboflavin Supplement, Thiamine Mononitrate, Copper Sulfate, Zinc Oxide, L-Lysine, Manganous Oxide, Ferrous Carbonate, Zinc Sulfate, Calcium Iodate, Cobalt Carbonate, Sodium Selenite.

[1]The vendor source was Purina

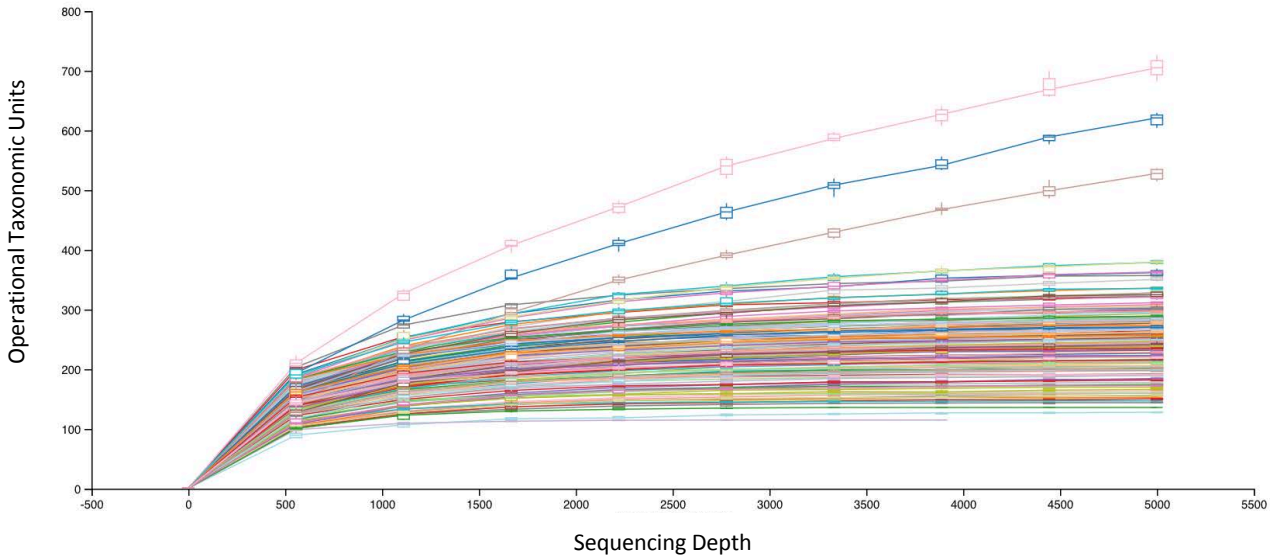
Supplemental Table 2. Mother and Infant Sample Descriptives

Mother

Time	*N*
Birth	12
2 Weeks	29
2 Months	27
4 Months	25
6 Months	23
Total	116

Infant

Time	*N*	*Female*	*Male*
Birth	12	7	5
2 Weeks	46	25	21
1 Month	16	10	6
2 Months	38	21	17
4 Months	44	22	22
6 Months	33	17	16
8 Months	29	11	18
10 Months	26	11	15
12 Months	25	11	14
Total	269	135	134



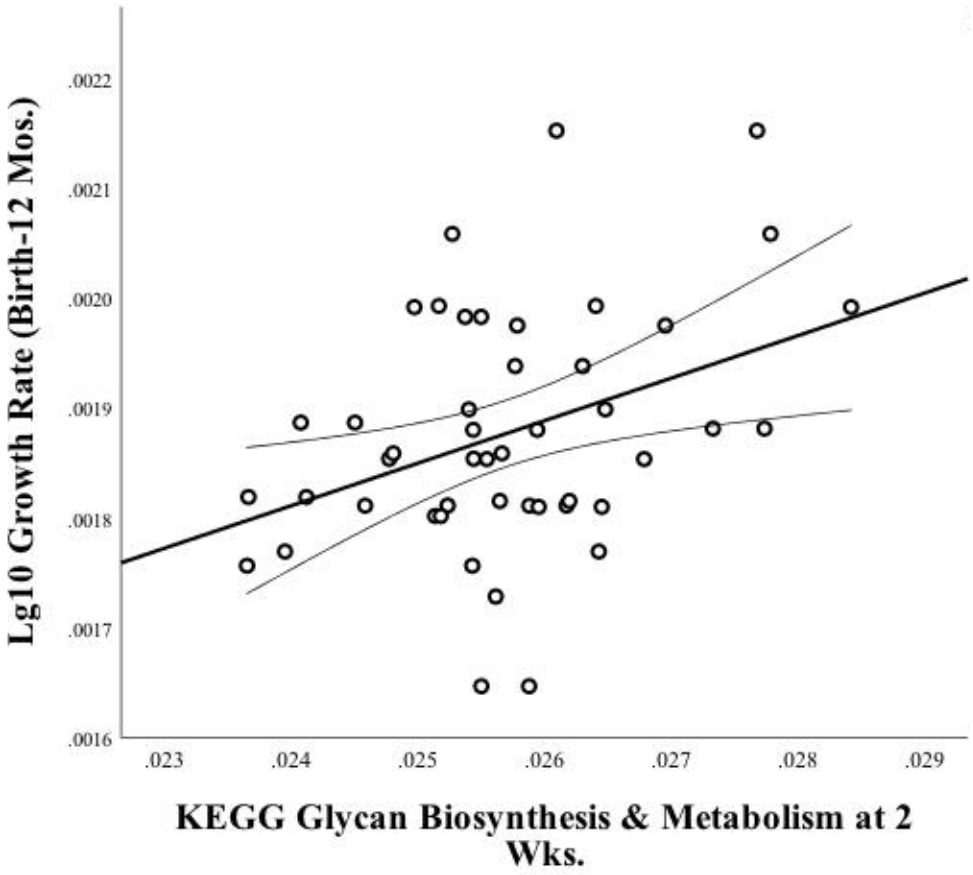
Supplemental Figure 1. Sample Alpha Rarefaction Plots: Rarefaction curves demonstrate that a sampling depth of 5000 sufficiently characterized the microbiome of our samples. The line chart in the upper figure connects the median values of the alpha diversity metric distribution across sampling depths. The box plots in the upper figure represent the distribution of the selected alpha diversity metric for sample at each even sampling depth. The lower and upper whiskers of the box plot are the 9th and 91st percentiles of the distribution (respectively), while the lower and upper extents of the box are the 25th and 75th percentiles of the distribution (respectively). The horizontal bar through the middle of the box is the median of the distribution (i.e., the 50th percentile).

Supplemental Table 3

Supplemental Table 3, KEGG Predicted Metagenomic Pathways at Level 2 for Mothers

OTU	Test-Statistic	P	FDR_P	Birth Mean	2wk Mean	2mo Mean	4mo Mean	6mo Mean
Membrane Transport	15.465	0.004	0.064	1015967.077	1082359.921	1361024.400	1389905.674	1253453.909
Xenobiotics Biodegradation & Metabolism	14.739	0.005	0.064	137884.000	146658.737	181683.900	185342.047	166878.909
Lipid Metabolism	13.258	0.010	0.064	232484.769	249708.474	307039.675	311582.581	279829.788
Transcription	13.173	0.010	0.064	237798.615	255987.132	312427.300	319299.674	295146.424
Carbohydrate Metabolism	12.878	0.012	0.064	883941.385	952041.763	1163273.250	1188064.860	1085255.273
Signal Transduction	12.779	0.012	0.064	128079.385	133360.868	172087.025	171531.861	146038.212
Immune System Diseases	12.749	0.013	0.064	4359.769	4745.789	5840.775	5997.558	5332.515
Signaling Molecules & Interaction	11.912	0.018	0.064	14073.615	15283.868	18002.050	18463.884	17504.697
Genetic Information Processing	11.556	0.021	0.064	238896.385	255373.184	312807.625	314018.302	282182.212
Poorly Characterized	11.531	0.021	0.064	419307.692	449987.763	550359.400	554053.302	498234.939
Cell Growth and Death	11.327	0.023	0.064	48482.538	52432.737	63595.650	64295.209	58469.879
Infectious Diseases	11.316	0.023	0.064	32709.462	35237.947	42652.050	43339.256	39196.788
Metabolism	11.262	0.024	0.064	200771.385	214831.447	262776.750	264169.209	237380.061
Translation	11.227	0.024	0.064	555672.769	594567.342	729668.675	732586.116	659811.152
Metabolic Diseases	11.138	0.025	0.064	8921.692	9620.184	11483.025	11659.907	10869.424
Replication and Repair	11.128	0.025	0.064	834225.308	895469.711	1088868.850	1097313.605	1000299.909
Amino Acid Metabolism	10.758	0.029	0.070	811589.385	872348.316	1064008.625	1068737.395	962426.970
Enzyme Families	10.667	0.031	0.070	192256.077	207390.921	248979.925	252099.930	231298.364
Nucleotide Metabolism	10.486	0.033	0.071	380612.539	411163.711	495558.700	500490.279	456680.576
Circulatory System	10.334	0.035	0.072	1194.231	1090.000	1965.425	1499.884	404.333
Metabolism of Other Amino Acids	10.164	0.038	0.074	127262.308	137642.632	165300.225	166925.070	151105.546
Excretory System	9.971	0.041	0.076	940.231	931.184	1168.025	1163.651	1037.061
Cardiovascular Diseases	9.811	0.044	0.078	34.769	27.842	52.750	37.581	25.212
Nervous System	9.638	0.047	0.078	8420.846	9114.000	10884.200	10984.256	10250.697
Metabolism of Terpenoids & Polyketides	9.584	0.048	0.078	148815.000	160983.158	193350.825	195030.442	176657.364

Environmental Adaptation	9.492	0.050	0.078	15162.154	16042.263	20312.725	20198.349	17441.303
Cell Motility	9.425	0.051	0.078	212966.846	218347.632	292881.425	284553.233	218146.818
Cellular Processes & Signaling	9.297	0.054	0.079	335875.077	361013.842	436657.225	438285.651	399487.697
Biosynthesis of Secondary Metabolites	9.097	0.059	0.083	74531.846	81201.605	95497.575	97379.860	89935.848
Folding, Sorting & Degradation	8.861	0.065	0.088	222302.846	238159.974	290182.075	289360.395	257649.818
Energy Metabolism	8.760	0.067	0.089	523615.308	561310.579	684747.575	683376.186	612831.485
Endocrine System	8.122	0.087	0.112	23026.385	24822.421	29347.925	29665.512	27466.152
Metabolism of Cofactors & Vitamins	7.908	0.095	0.118	378858.615	408958.237	489632.175	488338.977	443086.697
Transport and Catabolism	7.563	0.109	0.131	20381.692	22076.526	26221.725	25940.326	23367.091
Immune System	6.562	0.161	0.189	7829.385	8537.711	10172.750	10134.488	9065.636
Neurodegenerative Diseases	6.285	0.179	0.204	12446.538	12290.447	18272.950	15897.721	9601.515
Cancers	4.916	0.296	0.328	9881.692	10312.158	12939.325	12543.698	10685.788
Glycan Biosynthesis & Metabolism	4.839	0.304	0.328	206017.769	223863.632	262908.325	260115.721	232300.424
Digestive System	1.912	0.752	0.790	4612.692	5600.737	5370.625	5636.791	5693.182



Supplemental Figure 2. Correlation of the predicted activation of glycan biosynthesis and metabolism pathways at 2 weeks of age with infant growth trajectories, as determined through weight gain from birth to 12 months of age (r = 0.44, p= .003). Confidence intervals 95%

Chapter 3: Neurobehavioral correlates of the composition and sequential maturation of the gut microbiota

ABSTRACT

Background: Gut health has recently been suggested to play a role in the regulation of mood and behavior, particularly through the bidirectional communication pathways in the gut-brain axis. Infancy is a key period in the maturation of the gut microbiome and also the most dynamic period for postnatal neurodevelopment. Therefore, it is also a critical window during which gut-brain connections are established. Studies in humans have suggested that behavioral phenotypes in infancy are associated with gut microbial composition, however, there have been few longitudinal investigations into how these relationships arise over the course of development.

Methods: To investigate the association between infant temperament and neurodevelopment and the colonization of the gut, 24 rhesus monkeys (*Macaca mulatta*) were followed from 2 weeks after birth to 1 year of age. Three standardized assessments of behavior reflective of the development of autonomy, dietary transitions, and emotionality were conducted, and rectal swabs were collected at 2-month intervals. Microbial rRNA was sequenced with 16S Illumina MiSeq and measures of microbial compositional structure and the abundances of several major taxa shown to change with infant age were determined. At 12 months of age, infants underwent structural magnetic resonance imaging and gray matter and white matter volumes were quantified for total brain and major cortical regions associated with emotionality.

Results: In the first analysis, the progression of the microbiota colonization of the infant gut was evaluated and it was found that infant consummatory behavior and the development of behavioral autonomy were associated with the relative abundances of commensal taxa *Bifidobacterium* and *Lactobacillus*. More emotionally reactive temperaments were found in

infants with gut microbiomes that trended toward a less regulated acquisition of microbial richness along with alterations in the abundance of the taxa *Faecalibacterium*. The second analysis examined whether infant temperament phenotypes, and the accompanying shifts in microbial succession, were associated with neurodevelopment. Brain volume at 1 year of age was associated with a more even microbial structure during the peak period of nursing, characterized by a higher abundance of Firmicutes. More emotionally reactive infants had smaller hippocampal and cingulate regions of the brain, along with larger cerebellar volumes. These neural volumetric differences were associated with differences in the acquisition of microbial richness, however, an effect of microbial succession across the first year of life did not appear to mediate the influence of infant temperament on brain development.

Conclusions: Gut microbiome composition, including microbial community structure and the abundance of specific bacterial genera, were found to be associated with several aspects of behavior, temperament, and brain development in infant monkeys. This observational study provides further evidence that there are important relationships between the gut and brain that are established early in life.

What Is New:

- Abundances of *Lactobacillus* and *Bifidobacterium* within the infant gut were sensitive to dietary transitions from breast milk to solid foods and reflective of infant autonomy in a time-dependent manner.
- Abundance of *Lactobacillus* was unrelated to breastfeeding behavior.
- The gut microbiome of emotionally reactive infants was characterized by modest alterations in microbial richness and diversity and lower levels of the commensal *Faecalibacterium* post-weaning.
- Infants exhibiting larger brain volumes at 12 months of age had a more even microbiome during peak nursing, characterized by higher abundance of Firmicutes.

INTRODUCTION

Primates are colonized by more than 10 trillion microbes which reside in niches throughout the body, including the skin, oral cavity, and the gut [1]. These microscopic organisms collectively comprise the gut microbiota and are so numerous that their weight approximates that of the human brain [2]. As shown in Chapter 2, the gut microbiota is established early in life and its colonization involves a succession of taxa that is largely maternal in origin and reinforced through nutrients and prebiotic substances in breast milk. The gut microbiota vary widely across individuals, and have been implicated in the development, maturation, and maintenance of systems essential to health, such as metabolic functioning and the immune system [3,4]. In addition to affecting host physiology, there has been a recent surge of evidence indicating a central role for the microbial communities inhabiting the gut in regulating affect and behavior. We are now aware of the existence of a gut-brain axis, a bidirectional signaling pathway allowing for the top-down influence of the central nervous system (CNS) and emotional reactivity on gastrointestinal (GI) function and homeostasis, as well as a bottom-up modulation of the brain and hypothalamic-pituitary-adrenal (HPA) axis activation via immune, endocrine and neural mechanisms [5]. Gut microbiota exert influence on the gut-brain axis through a range of bioactive compounds, such as short chain fatty acids (SCFA), and through the synthesis of neurotransmitters, including serotonin and gamma-aminobutyric acid [6]. Perturbations in the gut-brain axis are apparent in the high comorbidity of GI tract and affective disorders, and have also been implicated in various developmental disorders, such as autism spectrum disorder, and later adult onset conditions characterized by neuroinflammation including Parkinson's and Alzheimer's diseases [7–10]. Given that

alterations in both domains appear to have cascading effects on one another, the normal establishment of the bidirectional crosstalk between the gut and the brain during early life may be essential for healthy developmental outcomes.

In primates, the first year of life is characterized by extensive structural and functional brain plasticity and calibration of the HPA axis. Evidence from animal models suggests that the presence of the gut microbiota during these critical windows of development is a prerequisite for normal neurodevelopment [11]. Over the last decade, it was observed that gnotobiotic rodents show alterations in HPA axis activation that manifest as deregulated stress responsivity, anxiety-like behaviors, sociality, and cognitive performance [12–15]. These behavioral abnormalities are accompanied by alterations in neurogenerative processes, including changes in the formation of the blood-brain barrier, synaptic transmission, microglial maturation, myelination, and neurogenesis [16,17]. Brain regions associated with the pathogenesis of anxiety appear to be especially impacted. For example, germ-free mice display aberrations in the myelination within the prefrontal cortex, in the metabolism and structural morphology of the amygdala and hippocampus, and in the dysregulated production of brain-derived neurotrophic factor (BDNF) and hippocampal serotonin [18–21].

Alternatively, the administration of probiotics, live organisms that provide health benefits when consumed, has been found to attenuate stress responsivity in both rodents and humans [12,22,23]. Colonizing neonatal germ-free mice with *Bifidobacterium infantis,* but not *Esherichia coli,* completely normalized the aforementioned HPA axis hyperactivity, and in rats, the administration of *Faecalibacterium* was found to minimize the glucocorticoid and inflammatory responses to an unpredictable stressor [12,24]. Furthermore, there is evidence

that neural plasticity is sensitive to input from the microbiota at specific stages during early life. As a result, bidirectional pathways of communication between the gut and the brain are increasingly difficult to establish later at older age [20]. For instance, while the microbial recolonization of the gut at three weeks of age was found to normalize anxiety phenotypes in germ-free mice, colonization in adulthood was insufficient to restore the altered behavioral phenotypes [21]. Taken together, these studies demonstrate that the presence of specific gut microbiota during key early developmental periods is essential for the establishment of appropriate stress responsiveness. Infancy likely represents a critical period for the establishment of these relationships as it is the most dynamic period of brain development and a sensitive time window for maturation of the gut microbiome.

While the bottom-up modulation of the gut microbiome on the CNS has only recently been discovered, it has long been known that behavior and affect, predominantly mediated through the HPA axis, can influence the composition of the gut microbiota. Over 40 years ago, Tannock and Savage reported alterations in endogenous microbiota, including lower levels of the symbiote *Lactobacillus*, following an environmental stressor [25]. More recent research expanded on these findings and indicated that stressor-induced activation of the autonomic nervous system, and the subsequent release of glucocorticoids and norepinephrine into the GI tract preferentially stimulated the growth of specific bacterial taxa and affected microbial adherence to the intestinal mucosa [26]. Stress also has a more indirect influence on microbiota composition. Stress-related changes in signaling via the enteric nervous system or the vagal nerve alter GI motility and reduce digestive activity, which impact the gut environment and metabolic substrate availability [27]. Additionally, activation of the autonomic nervous system

diverts blood away from the GI tract [28]. In doing so, oxygenation of the intestinal mucosa is diminished, ultimately degrading the physical barrier of the gut lining and increasing paracellular permeability within the intestinal epithelium. Increases in gut permeability contribute to the capacity of bacteria to translocate outside of the gut and induce inflammatory responses within the host, which in turn further alter the intestinal environment and contribute to the intestinal dysbiosis [29].

Evidence for these mechanistic pathways has been obtained in both human and animal studies, where environmental, physiological, and psychological stressors have all been demonstrated to alter the composition of the gut microbiota [27,30,31]. If experienced during early periods of developmental plasticity, immune activation and gut permeability are especially problematic. The neonatal brain is more vulnerable to circulating toxins due to the greater fragility of the developing cerebral vasculature [17]. Similarly, exposure to psychological stress during development may have an enduring effect on host physiology, impacting the gut environment long after cessation of the stressor. For instance, adult rats that had experienced separation from the mother as pups, an early-life stressor, display a depressive phenotype, increased signs of inflammatory activity, coupled with reductions in certain commensal taxa [32]. These findings suggest that activation of the HPA axis due to early-life stress may produce both immediate and long-term changes in stress responsiveness and gut microbiota composition.

While aware of the existence of bidirectional communication between the gut and the brain, we are still largely ignorant of how these pathways are established during development. The extant research on the developmental origins of the gut-brain axis has primarily employed

models with germ-free mice, but differences in neurodevelopment, as well as the fact that humans are never in a truly germ-free state, limit the translational relevance of those findings [11]. Research with primate models, such as the infant rhesus monkey, may provide complementary information that can deepen our understanding of gut colonization during infancy. In Chapter 2, it was shown that nonhuman primates display some similarities with human infants in gut colonization during the first year of life. Their stress-related endocrine function and behavioral development trajectories have also been extensively studied [33]. However, prior studies that demonstrated psychosocial influences on the gut microbiome of young monkeys have focused more on older juvenile monkeys that were at least 6 months old [34,35]. Because both the brain and the gut microbiome are most plastic in early development, it is also important to investigate the influence of early-life experiences on the microbial progression of the gut beginning soon after birth.

This study addressed these gaps in our understanding by conducting a series of analyses to provide more insight into the complex interplay between gut microbiota colonization, infant temperament, and rearing in the development of the gut-brain axis and calibration of stress reactivity. In the first phase of the study, rhesus infants were followed over the first year of life during which microbial profiles were examined and infant emotionality and development characterized through standardized behavioral assessments and observations of mother-infant interactions. The second phase of this analysis examined whether infant temperament, and the accompanying shifts in microbial succession, corresponded to neurodevelopmental outcomes, as determined through magnetic resonance imaging of brain regions associated with

emotionality. Collectively, these experiments employed a longitudinal approach that helps to better clarify the existence of critical developmental windows for the gut-brain axis.

Analysis 1: Temperament, Early-life Experiences & Gut Microbial Trajectory

In the first analysis, the progression of the gut microbiota colonization was evaluated in conjunction with assessments of infant behavior. In primates, differences in infant behavior can be assessed through identification of temperament phenotypes, which refer to biologically-based individual differences in dimensions of behavior, specifically, in emotionality, self-regulation, and activity [36]. Temperament emerges early in infancy, and despite continuing to be a stable feature throughout the lifespan, the development of temperamental phenotypes is sensitive to the early environment and to interactions with the caregiver [37–39]. Significantly, certain temperament characteristics in humans are closely associated with developmental and mental health outcomes. For instance, an anxious temperament in early life may continue to be manifest as either excessive behavioral reactivity or behavioral inhibition, and these dimensions of emotional reactivity have been found to precede the emergence of anxiety and depressive disorders [40]. Moreover, temperament characteristics have a physiological basis. In both humans and rhesus monkeys, behaviorally reactive and inhibited temperaments have been associated with heightened amygdala activation and a dysregulation of the HPA axis, which are risk factors for later psychopathology [36,41]. As previously noted, higher stress reactivity and poor emotional regulation have been linked to aberrations in the intestinal physiology and diminished diversity and richness of the gut microbiota [42]. Therefore, it was hypothesized that the establishment of a more emotionally reactive temperament early in life, and greater

stress responsiveness, could be associated with the developing microbiota during infancy, evinced by a different microbial succession during the nursing period and diminished bacterial diversity after weaning and the transition to solid foods.

In species with extended postnatal care, such as the primate, maternal-infant interactions also have the potential to influence infant stress reactivity and the formation of the gut-brain axis through several routes. Following the initial inoculation of bacteria during the birthing process, the mother continues to contribute to the neonate's acquisition of gut microbes through direct skin-to-skin contact and through breastfeeding, which provides critical nutrients to the colonizing bacteria and is itself a source of microbes [43,44]. The degree to which mothers interact with their offspring is, therefore, likely to directly impact how bacteria proliferate and are sustained in the gut of the infant. Mother-infant interactions may also impact the infant microbiome through more indirect means. In addition to seeding the infant microbiota, mothers provide responsive caregiving and tactile stimulation that underlie attachment security and promote the infant's emotion regulation [45,46]. Differences in the quality of maternal care and maternal solicitousness could have enduring influences on brain development and stress responsiveness to environmental challenges. In rodents, naturally occurring changes or reductions in maternal care were found to result in dysregulated HPA activation in offspring [47], which in turn, may alter the stability of beneficial bacterial taxa. This type of influence from a disturbance of the mother-infant relationship was also demonstrated by Bailey and Coe when 3 days following separation from the mother, there was a significant decrease in *Lactobacillus* in infant rhesus monkeys, which corresponded with elevations in stress-related behaviors and an increase in diarrheic symptoms due to *Campylobacter jejuni*

[34]. Therefore, the commensal taxa associated with breastfeeding, as well as the more abundant taxa in the maternal gut, such as *Prevotella*, were hypothesized to be diminished in more emotionally reactive infants and those receiving less solicitousness care from their mothers.

Finally, the time to reach typical developmental milestones could be impacted by infant temperament and might be associated with the pattern of microbial succession in the infant gut. In Chapter 2, the infant's diet appeared to be a major determinant of gut microbial structure with the transition from breastmilk to the consumption of solid foods dramatically shifting the infant gut to a more adult-like microbial community. Among humans, it has been reported that infants with a more 'difficult' temperament are breastfed for shorter durations and are introduced to solid foods before the pediatrician-recommended age [48]. Alternatively, behaviorally inhibited infants tend to show negative affect and withdraw from novel stimuli, and this aversion to novelty has been observed to translate to a neophobic reaction to unfamiliar foods [49]. Within the current study, it was hypothesized that infants with emotionally reactive temperaments might exhibit a difference in consummatory behavior and transition to solid foods at a different age. An insecure attachment and emotionally reactive temperament may also influence when the infant is first exposed to bacteria in the physical environment. Without a 'secure' base from which to explore their environment, these infants may interact with their surroundings at lower rates or at an older age [50], thereby diminishing the acquisition to other bacterial taxa from their rearing environment. Conversely, infants who become independent and transition to solid foods at a younger age were hypothesized to host

a more diverse gut microbiome characterized by a lower abundance of the oligosaccharide consumers *Bifidobacterium* and *Lactobacillus.*

ANALYSIS 1: HYPOTHESES & PREDICTED RESULTS

Hypothesis 1.1: Emotion regulation is hypothesized to be stable over infancy and indicative of temperament. In turn, temperament phenotypes will be associated with microbial diversification, as indicated by the richness and evenness of microbial community structure, and the rate of microbial acquisition.

Predicted Results 1.1: Behaviorally reactive and inhibited temperaments will be predictive of diminished abundance of *Lactobacillus* and *Bifidobacterium* and correspond to atypical acquisition of an adult microbiome community structure.

Hypothesis 1.2: Microbial succession will closely reflect the timing of dietary transitions and the amount of solicitous caregiving received by the infant.

Predicted Results 1.2: Lower alpha diversity and higher abundances of the oligosaccharide consumers *Bifidobacterium* and *Lactobacillus* will reflect differences in nursing behavior. Conversely, these commensal taxa and the abundance of Prevotella abundances, will be lower in emotionally reactive infants and those with less solicitous mothers.

Hypothesis 1.3: During the nursing period, the development of infant autonomy and emotional regulation will be predictive of increased exposure to environmental microbes and an earlier acquisition of a more adult-like gut microbial community structure.

Predicted Results 1.3: Emotion regulation and independence from the mother will affect the diversity and balance of the microbial communities, especially the more abundant commensal taxa.

ANALYSIS 1: METHODS

Subjects. The subjects were 24 mother-infant pairs of rhesus monkeys (*Macaca mulatta*; 12 female) born and reared at the Harlow Center for Biological Psychology. Infants were born between 2016 and 2018 and were followed from 2 weeks postnatal to 12 months of age. Animals were housed separately with just the mother or in paired mother-infant dyads until around 6 months of age and were then weaned into groups of 3-6 similarly aged infants to facilitate normal socialization and provide companionship. Peer groups were housed in larger pens or runs (3.2 × 0.8 × 0.8 m). Monkeys were fed the same diet manufactured for primates (Lab Diet 5LFD, St Louis, MO), and were supplemented with fresh fruits and vegetables. Only healthy animals without exposure to antibiotics during the first year of life were used. All animals were in visual and auditory contact with other monkeys and received extensive enrichment. Routine husbandry procedures and veterinary care at the Harlow Center for Biological Psychology (HCBP) meet and exceed the recommendations of the Guide for the Care and Use of Laboratory Animals. This facility is inspected by institutional Animal Care and Use Committee (ACUC) on a semi-annual basis as well as by the USDA, and records are regularly reviewed. Husbandry and experimental procedures were reviewed and approved by the ACUC.

BEHAVIORAL ASSESSMENTS

An infographic of the study timeline and behavioral assessments is provided in Figure 1.

Infant Behavioral Assessment Scale. Infant neurodevelopment and behavioral reactivity were evaluated with the standardized Infant Behavioral Assessment Scale (IBAS), which was modeled from the Brazelton Neonatal Assessment scale for human infants. The test was administered at 2 weeks of age, which has been demonstrated to be a more reliable assessment than timepoints closer to birth and also representative of subsequent responses at 3 and 4 weeks of age [51,52]. Infants were removed from their mothers for approximately 20 minutes between 0930-1100. The scale evaluates a total of 29 attributes that are known to mature over the first year of life, and include measures of emotionality and arousal, attention reactions to visual and auditory cues, responses to vestibular and tactile stimulation, and neuromotor reflexes [53]. The same experimenters, blind to subject condition, always administered the scale to ensure reliability. Further details about the IBAS and the detailed definition of each item have been previously described [51].

Mother-Infant Observations. Observations of mother-infant interaction began around 2 months following birth and continued until the infant was 4 months of age, a dynamic and sensitive period in infant development. Defined behaviors characteristic of mother-infant dyad interactions were observed and scored for 5-minute intervals, once per day, 12 times per month. Each month's 12 behavioral records were averaged to generate representative 300 second monthly scores for the mother-infant dyad. Maternal solicitousness and infant

autonomy were determined using the frequency and duration of behaviors specified by Shirtcliff, Phan, Lubach, Crispen, & Coe (2013) [54]. The degree of maternal solicitousness was measured through the duration of mother-infant contact and grooming of the infant and the frequency of the mother re-establishing contact. An infant's degree of autonomy was established through frequency of the infant reestablishing contact with the mother and the duration of infant activity and environmental exploration. Infant vocalizations were additionally evaluated as a measure of emotional reactivity. The duration of time spent breastfeeding, as determined through time spent in contact with the nipple, and the initiation of consumption of solids was also considered because infant diet is understood to be major determinant of the gut microbial composition. Linear Mixed Models were used to examine developmental changes in alpha diversity indices with respect to dyad behaviors, and linear regression with false detection rate adjustment was used to evaluate taxa and behavior from a single time point.

Human Intruder Paradigm. Between 11-12 months of age, infant rhesus were evaluating using the Human Intruder Paradigm (HIP), a well-validated assessment used to measure an individual macaque's response to an unfamiliar human intruder, which serves as a potentially threatening social stimulus [55]. In the HIP, monkey subjects are separated from their cage-mates and relocated to a separate testing room out of hearing range of other macaques, with the goal of eliciting distinct fear and anxiety-related behaviors. The test is recorded on a video camera and consists of 3 two-minute baseline periods, interspersed with a 2-minute period where the human intruder stands with their profile facing the monkey (No Eye Contact condition), and a 2-minute Stare condition, where the intruder faces the monkey and makes direct eye contact. A

baseline period separates the profile and stare conditions to reduce habituation to the intruder. For further detail on the social threat conditions within the HIP, as well as a description of normative responses, see Supplemental Text 1.

The individual components of the HIP were evaluated to ensure that the assessment was sensitive to variations in behavioral responses, and also to determine component correspondence with the behaviors measured in the IBAS and during the mother-infant dyad observations. Composite variables were then created to reflect the latent factors previously reported to reflect differences in emotionality and in response to fear [41,56,58]. A description of the construction of the composite variables can be found in Supplemental Text 3.

Specimen Collection & DNA Isolation. Rectal swabs were collected with sterile culturettes (BBL Culture Swab Collection and Transport System, Becton Dickinson, Cockeysville, MD) at 2 weeks of age and at 2-month intervals until infants reached one year of age. Swabs were collected in the morning between 0930-1130 and were frozen and stored at -70 °C in an ultracold freezer. The DNA isolation protocol is included in detail in the previous chapters. Briefly, DNA was isolated using the PowerSoil SNA Isolation Kit (MoBio, Carlsbad, CA) and the purified genomic DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA). DNA then underwent PCR amplification of the V4 variable gene of the 16S rRNA gene using region-specific primers (515F-806R) and amplicon sequencing was performed by the Argonne National Laboratory (Argonne, IL) on an Illumina MiSeq Platform (Illumina, San Diego, CA).

Bioinformatics and Statistics. Forward and reverse reads were imported into QIIME2-2019.7, a plugin-based system that wraps independent microbiome analysis methods. Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin, and then underwent denoising with the DADA2 (q2-dada2) analysis pipeline, which detects and corrects observed amplicon sequence variants. Taxonomy was assigned to unique amplicon sequence variants (ASVs) using a Naïve Bayes filtered classifier (q2-feature-classifier) trained on the Greengenes database, version 13_8, at 99% sequence similarity [59]. Mitochondrial and chloroplast derived sequences were filtered out based on taxonomic annotation. Following rarefaction to a depth of 5000 sequences per sample, the QIIME2 q2-diversity plugin was used to produce weighted and unweighted Unifrac measures of beta diversity, as well as 3 measures of Alpha Diversity, including Faith's Phylogenetic Diversity, Pielou Evenness, and observed species (OTUs), which were previously described in Chapter 2.

Significance was tested by implementations in QIIME2 and R software (version 3.4.2). For multiple comparisons of measures of bacteria, threshold levels of significance ($p \leq 0.05$) for Spearman-rank correlation coefficients were adjusted by the Benjamini-Hochberg False Discovery Rates correction (FDR) [60]. However, because of the exploratory nature of these analyses, weaker trends at $p \leq 0.075$ were also denoted. Wilcoxon rank sum test (the nonparametric equivalent of the t-test) was used to evaluate effects of temperament on the relative abundances of individual microbial taxa at a single timepoint. PERMANOVA tests with 999 permutations were used to determine whether between-sample distances were significantly different between temperament phenotypes. Longitudinal analysis was conducted on beta and alpha diversity and the relative abundances of dominant taxa using the Q2-

longitudinal plugin and linear mixed-effects modeling (LMM) [61]. Briefly, to assess the temporal dynamics of microbial metrices as a function of infant temperament, each metric was regressed on infant age and its interaction with infant temperament using restricted maximum likelihood (REML) with a random-intercept by subject to account for repeated measurement. Microbial analyses were limited to phyla and genera that made up at least 1% of the total sample by relative abundance and were shown in Chapter 2 to undergo maturational changes in abundance. It has been previously reported that less abundant genera may have reduced functional input [62], and by decreasing the number of comparisons made to those between temperament and only dominant, highly variable taxa, statistical power is increased.

EXPERIMENT 1: RESULTS & DISCUSSION

Infant Behavioral Assessment Scale (IBAS). The IBAS was used to examine early variation in infant behavior, responsiveness, and neuromotor development. Five IBAS variables were excluded from the analysis due to limited variance in the scores. The remaining 24 IBAS variables were collapsed into four composite scores following data from a previous factor analysis in infant rhesus monkeys of the same age [53]. The four composite scores include (1) State Control, (2) Sensory Sensitivity (3) Motor Maturity, and (4) Orientation. Congruent with previous reports, male and female infants did not differ significantly on any IBAS subscale [63]. These composite scores and their individual components were evaluated with respect to infant gut microbial alpha diversity metrics and the prevalence of eight abundant genera at 2 weeks of age that were identified in Chapter 2 to vary significantly throughout nursing. These taxa

included *Prevotella*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Flexispira*, *Catenibacterium*, *Faecalibacterium*, and *Bacteroides*.

The State Control factor assesses early temperament and was measured through infant reactivity and state regulation during the administration of the orienting and neuromotor items. An accelerated acquisition of a phylogenetically diverse and abundant microbiome at 2 weeks of age was predictive of more displays of emotional reactivity in infants (r=.48, p= 0.019; Figure 2A). Furthermore, increased abundances of OTUs within the gut microbiome were associated with the infant exhibiting more fearfulness (r=.31, p= 0.063; See Supplemental Materials, Table 1 for complete IBAS & Microbial Correlations), more struggling during the test (r=.33, p= 0.05), and less soothability, which refers to an infant's tendency to show reductions in distress in response to calming techniques employed by the experimenter (r=-.36, p= 0.029). More difficulty to regulate emotion was also apparent in infants hosting GI tracts enriched in the genera *Streptococcus*, within the Firmicutes phylum, which at 2 weeks of age is a strong indicator of phylogenetic diversity (r=.45, p= 0.004). These infants made more vocalizations, responded more intensely to stimuli, and struggled throughout administration of the IBAS *(*r=.33, p= 0.047; r=.41, p= 0.031; r=.31, p= 0.06, respectively). While our findings indicate that an excessively diverse and abundant microbiota at 2 weeks of age is characteristic of a more emotionally reactive infant, there was no observable relationship between infant temperament and the evenness of the microbial composition.

A diverse microbiome is generally a signal of a healthy microbiome; however, this is not the case in early infancy. The oligosaccharides in breastmilk preferentially promote the predominance of the Actinobacteria *Bifidobacterium* and inhibit the growth of potentially

pathogenic strains, thereby suppressing gut microbial diversity. Though not significantly predictive of the State Control factor, *Bifidobacterium* was inversely correlated with response intensity and scores of fearfulness *(r*=-.33, *p*= 0.048; *r*=-.38, *p*= 0.019, respectively). Infants hosting higher populations of *Bifidobacterium* also displayed more self-calming behavior (*r*=.37, *p*= 0.024); and trended toward less extreme responses to tactile stimulation *(r*=-.30, *p*= 0.065), both measures indicative of greater emotional regulation. These findings are in keeping with the research on human infants, which has reported the presence of *Bifidobacterium* may have a central role in inhibiting inflammation and in reducing infant fussiness and crying [64]. In addition, levels of this genus are reported to be suppressed in infants exhibiting colic [65].

In addition, a more even and balanced gut microbial composition trended toward higher scores on the Motor Maturity IBAS factor *(r*=.31, *p*= 0.067), which measured muscle tone, coordination, balance, and spontaneous movement through normative reflexes, motor activity, and speed of responding. As described in Chapter 2, the infant gut is rapidly transitioning during the first several weeks of the life, and the predominance of *Bacteroides* seen in the neonatal gut is largely replaced by a more evenly distributed composition characterized by elevated abundances of *Prevotella* and *Bifidobacterium*. Within the current study, higher levels of *Prevotella* at 2 weeks were indicative of a more balanced microbiome and were independently correlated with several components of the Motor Maturity factor (See Figure 2B), including coordination *(r*=.43, *p*= 0.008) and motor activity (*r*=.45, *p*= 0.005). The opposite relationship is seen with elevated levels of *Bacteroides (r*=-.45, *p*= 0.005; *r*=-.38, *p*= 0.028, respectively). Previous studies have reported that members of the Bacteroidetes phylum are generally environmentally determined [66,67], which suggests that acquisition of *Prevotella*, and

consequently the attainment of a more balanced gut microbiome, is paralleled by the maturation of motor responses.

Taken together, our findings demonstrate that the changing microbial profile at 2 weeks of age generally accompanies differences in infant motor and emotional reactivity, as measured with the IBAS test. It was not surprisingly that the genera implicated in these associations were the same taxa in Chapter 2 to be undergoing major transitions from birth to 2 weeks of age, i.e., *Bacteroides*, *Prevotella*, and *Bifidobacterium*. In contrast, there was not a consistent relationship with the microbial diversity metrics for the Sensory Sensitivity or Orientation scores, which captured the infant's reactions to vestibular and tactile stimulation and assessed tracking of visual and auditory stimuli respectively. Nor was there an association between any behaviors scored with the IBAS and the abundance of *Flexispira*, *Faecalibacterium*, or *Catenibacterium*.

Mother-Infant Observations. Interactions between mother-Infant dyads were observed between 2-4 months of age to evaluate infant autonomy, the transition to independent feeding behavior, and maternal solicitousness. Repeated measures ANOVAs indicate that, as expected, the behavior of the infant and mother underwent significant maturational changes across the first 4 months following birth. The amount of time the dyad spent in contact at 4 months of infant age declined by nearly 34% from that recorded at 2 months of age, $F_{(2,46)}=9.12$, $p=.001$. In keeping with the decrease in physical contact, infants spent progressively more time exploring their environment, $F_{(2,46)}=6.59$, $p=.003$, and mothers retrieved their offspring less often as infants became more independent by 4 months of age, $F_{(1,23)}=6.84$, $p=.015$. Infant diet

similarly transitioned, with time spent breastfeeding at 4 months decreasing to 52% of the duration recorded at 2 months, $F(_{2,46})= 31.21$, $p<.0001$. All but 7 infants had begun sampling solid foods by 2 months of age. Sex differences in both the infant's behavior and maternal responsiveness to their infants were not detected.

Mother-infant dyad behaviors were evaluated with respect to alpha diversity metrices and to 5 abundant taxa measured at 2 weeks and 2-4 months postnatal (*Prevotella*, *Lactobacillus*, *Streptococcus, Bifidobacterium*, *Faecalibacterium*, *Catenibacterium*, and *Flexispira*). Because *Bacteroides* had diminished to <1% by 2 months of age, those levels were not evaluated with respect to the infant's behavior. Our findings from Chapter 2 demonstrated that the progressive changes in the microbiome of the rhesus infant are generally consistent with the human literature. Specifically, the period of infancy is characterized by reduced microbial diversity within the gut. As infant development progresses, microbial diversity is acquired though environmental exposure and is largely dependent on infant diet, i.e., breastfeeding, formula-use, or the consumption of solids. Thus, a divergence from the predicted microbial succession, either through accelerated or delayed microbial diversification, was hypothesized to parallel differences in the timing of transitioning feeding behaviors and in the onset of more autonomy.

In human infants, breastfeeding has been reported to be a major determinant of reduced diversity through both the extended contact it provides infants with their mother and through increased exposure to prebiotic substances in breast milk, which foster the growth of the commensal genus *Bifidobacterium* [68]. Unexpectedly, the duration of breastfeeding, as determined through oral contact with the mother's nipple, was not a determinant of reduced

phylogenetic diversity and richness within the gut microbiome of the infant monkey (See Supplemental Materials, Table 2A). It was found, however, that more time spent in contact with the mother's ventrum and observed on the nipple over the 2nd and 3rd-month observations corresponded to a higher abundance of *Bifidobacterium* across the first 4 months of life (See Figure 3A & Supplemental Materials, Table 2B). The absence of an effect of breastfeeding on microbial diversity within the infant gut may be the result of rhesus monkey breastmilk containing fewer oligosaccharides than human milk [69]. This species difference would suggest that the milk of rhesus monkeys induces less of a competitive advantage to *Bifidobacteria* and bacteria that produce lactic acid, thereby allowing for the colonization of a more diverse microbiome. This finding is reflected in the results reported in Chapter 2 that indicated, for the majority of infancy, *Bifidobacterium* is not the dominant genus in the rhesus gut. Despite differences in the oligosaccharide content of non-human primate milk and the absence of an overt influence on gut diversity, an enduring effect of breastfeeding was still evident on the infant microbiome. Infants who spent more time nursing and had higher abundances of *Bifidobacterium* at 2 and 4 months of age were less likely to have begun transitioning to solid foods over this 2-month period (*Bifidobacterium* at 2 mos.: $F_{(1,22)}= 5.53$, $p=.024$; 4mos.: $F_{(1,22)}= 6.064$, $p=.022$, See Figure 3B).

Furthermore, the consummatory behavior of the infants and infant autonomy were intertwined over the first several months. Because breastfeeding requires the infant to be in contact with the mother, lower levels of independent locomotion and exploration of the environment at 2-3 months were predictive of a higher abundance of *Bifidobacterium* at 4 months of age ($r=-.43$, $p=.006$; $r=-.48$, $p=.002$, respectively). However, this lower infant

autonomy was transient and, by 4 months of age, infants with more *Bifidobacterium* early in life explored and engaged with their environment more actively (r=.43, p=.039).

In contrast, infants who adopted a more adult-like diet of solid foods at a younger age hosted a richer and more phylogenetically diverse gut. Specifically, more time spent consuming of solid foods at 2 months of age was associated with elevated Faith's Phylogenetic Diversity at both 2 and 4 months of age (2 mos.: r=.43, p=.015; 4 mos.: r=.31, p=.047). In comparison to infants still exclusively breastfeeding over the 2-month observations, infants that had started to sample solids exhibited a faster rate of acquisition of observed OTUs throughout the observation period ($F_{(2,46)}$= 3.71, p=.062). Genera within the Firmicutes phylum are indicators of microbiome maturation and generally become more dominant in the gut as nursing declines and more consumption of biscuits was observed. Within our sample, exploration of solid foods in the 2nd and 4th month of observations corresponded to a higher abundance of *Faecalibacterium* at 2 months and of *Lactobacillus* at 2 and 4 months (See Supplemental Materials, Table 2B). *Faecalibacterium* plays a critical role in the digestion of complex carbohydrates and has been found to increase at the cessation of breastfeeding in human infants [70]. Moreover, the finding that infant microbial composition at 2 months of age was a predictor of dietary behavior at later time points suggests that dietary changes and transitions in the gut microbial are related. The bacterial composition must transition from Actinobacteria to Firmicutes in order for infants to better utilize nutrients in solid food.

The finding that *Lactobacillus* abundance was associated with transitioning to solid foods replicates our previous report in Chapter 2 showing that the levels increase most dramatically around the time of weaning. In contrast to human infants, where *Lactobacillus* is

abundant early in vaginally-born infants and levels of lactic-acid producing bacteria are enriched through breastmilk [71], in our monkeys nursing was inversely associated with *Lactobacillus* abundance. At 4 months of age, more nipple contact *(r*=-.32 *p*=.042) and ventrum-to-ventrum contact *(r*=-.37, *p*=.019) predicted a lower abundance of *Lactobacillus*. Thus, the infant's increasing autonomy was reflected by an increase in *Lactobacillus* abundance. Higher *Lactobacillus* abundance at 2 months of infant age was associated with more time spent locomoting independently (2 mos.: *r*=.36, *p*=.02; 3 mos.: *r*=.36, *p*=.023), more exploration of the environment (4 mos.: *r*=.34, *p*=.035), and less frequent reestablishment of contact with the mother at 2 and 3 months of age (*r*=-.33, *p*=.038; *r*=-.36, *p*=.022, respectively).

In addition to the genera within Firmicutes, *Prevotella* within the phylum Bacteroidetes, was also present at higher levels in infants who more frequently sampled solid foods at 2 months of age (*r*=.33, *p*=.041). This effect remained at 4 months, with infants who began transitioning to solids before 3 months of age hosting gut microbiomes that were on average 7% more abundant in *Prevotella*. Like *Faecalibacterium*, *Prevotella* in humans is associated with a diet rich in complex fiber [72], such as the one provided by monkey biscuits. In keeping with the IBAS results, *Prevotella* abundance was additionally associated with infant exploration and activity at 2 months of age (*r*=.38, *p*=.018). This relationship between the level of *Prevotella* and motor maturity may reflect the fact that access to monkey biscuits requires more advanced motor skills than those required to breastfeed.

Microbial composition during early life was also associated with certain aspects of infant temperament that were observed during the mother-infant observations. Infants who were consistently more vocal (i.e., indicative of reactive emotionality) throughout the 4-month

observation period also hosted a more phylogenetically diverse, yet unevenly distributed microbiome, which was enriched with *Streptococcus* at 2 weeks of age (2 mos.: r=.51, p=.001; 3 mos.: r=.47, p=.002; 4 mos.: r=.32, p=.041). These findings concur with those reported earlier for infant behavioral responses during the IBAS. A more accelerated acquisition of a phylogenetically diverse and abundant microbiome at 2 weeks of age also corresponded with more frequent attempts by the infant to reestablish contact with the mother at 2 months of age (r=.35, p=.03) and less independent activity observed at 2 and 3 months (r=-.32 p=.048; r=-.29 p=.072, respectively). Conversely, a more diverse microbial community at 2 months of age and older, or a more evenly distributed microbial composition at any timepoint, were not predictive of delays in becoming more independent of the mother. A more even microbiome at 2 months of age was also associated with less vocalizing at all time points (2 mos.: r=-.63, p<.001; 3 mos.: r=-.60, *p<.001*; 4 mos.: r=-.42, p=.009) and more locomotory and exploratory behavior at 4 months (r=.47, p=.023). This pattern of behavior suggested that atypical microbial succession within the gut, such as an early colonization by an overly diverse microbiota, may precede the development of a more reactive temperament and impact the relationship between the infant and its mother.

However, variation in maternal solicitousness was not a significant predictor of microbial composition in the infant gut. The influence of maternal solicitousness on the microbial succession in the infant gut was evaluated through the duration of mother-infant contact, the amount of maternal grooming, and the frequency of mother re-establishing contact when the infant was out of reach. Infants who were in more sustained contact with their mothers did exhibit a less evenly distributed gut microbiome at 2 months of life (r=-.39,

p=.015). However, neither the frequency of maternal retrievals nor the duration of grooming were reflective of the infant's microbial status. It is possible that the behavioral measures of maternal solicitousness employed were not sensitive enough to capture differences in the mothers' responsiveness or that these aspects of maternal care do not affect the infant's gut physiology and gut bacteria.

Human Intruder Paradigm (HIP). Between 11 and 12 months of age, the emotionality of the older infants was evaluated in a standardized and quantitative manner using the Human Intruder Paradigm (HIP). The results from this test confirmed previous papers on the response of monkeys to the 3 HIP test conditions, which is that the infant's reaction varies depending on the presence and facial orientation of human observer in the room [55,73]. During the baseline period, monkeys spent significantly more time exploring their surroundings and pacing and were significantly less likely to freeze (*p*<.001, *p*=.042, and *p*=.01, respectively). During the condition when the human intruder presents their facial profile to the monkey, locomotion decreased significantly (*p*<.001), and infants spent more time freezing at the rear of the cage (*p*<.001 and *p*<.001). In contrast, during the stare condition when the human intruder makes direct eye contact with the subject, the monkeys exhibited more reactive behaviors. They oriented their body more frequently toward the intruder (*p*<.001) and vocalized more barks and screams, and showed fearful lip-smacking behavior (*p*=.001, *p*=.027, and *p*=.064, respectively). Coos and fear grimaces occurred only in the stare condition. In addition, the majority of threatening cage shaking behavior occurred during this third phase of the test. Female monkeys spent more of the stare condition located at the rear of the cage (*p*=.046), but

otherwise there were no sex differences in rhesus infant responses to this test paradigm. The distinct responses to each test condition indicate that the test paradigm was successful in capturing the monkeys' emotional reactions to social threat and the visual signal of staring.

Concordance of HIP Results with IBAS & Mother-Infant Dyad Observations. A detailed description of the congruence between the behavioral responses to the HIP and components of the earlier behavioral assessments at 2 weeks of age in the IBAS, and the observations of the mother-infant behavior is provided in Supplemental Text 2.

Hip Temperament Phenotypes & Microbial Succession. After confirming that the HIP captured distinct differences in infant emotionality, three categorical variables were created from HIP test scores to reflect several constructs suggested to underlie behavioral responses to a social threat; namely behavioral inhibition, behavioral reactivity, and inactivity [38,56,58]. Infant activity levels were assessed because animals who are behaviorally inhibited have been described as showing less activity in the absence of threatening stimuli [58]. Further description of the construction of the HIP temperament phenotypes is provided in Supplemental Materials, Text 3 and composite factor correlates are provided in Supplemental Materials, Table 3.

The HIP temperament phenotypes were employed to investigate the association between infant emotional reactivity and the colonization of the infant microbiota. Microbial metrices were assessed two ways. First, the rate of acquisition of a phylogenetically diverse and abundant microbiome and of developmentally-relevant microbial taxa within the infant gut were evaluated in relation to emotionality over the first year of life. Then, microbiome indices

that were acquired more contemporaneously with the administration of the HIP test were investigated with respect to monkeys' behavioral responses.

The infant gut microbiome is dynamic, and as shown in Chapter 2, the metrices indicative of microbial richness and diversity increased in parallel with infant age. Given that infant emotionality may potentially influence environmental exposure to microbes, it was hypothesized that a more reactive temperament would be associated with atypical microbial diversification. While not statistically significant, infants with behaviorally reactive and inhibited temperament phenotypes did exhibit deviations in the rate at which this microbial richness was acquired. Behaviorally reactive infants trended toward a less linear acquisition of microbial phylogenetic richness (REML FPD: p=.053, Figure 4A), characterized by higher microbiota community diversity following weaning. Conversely, more inhibited infants trended toward hosting increased microbial richness throughout the nursing period (REML Observed OTUs: p=.066, Figure 4B). The evenness of the microbial distribution was not found to be predicative of differences in HIP profiles. This may be due to our previous finding that evenness is acquired early in infancy and displays little temporal variation after the infant reaches 4 months of age. There was additionally no effect of having a behaviorally inhibited or reactive temperament on the temporal changes seen within infants' beta diversity, though active infants were found to exhibit a higher degree of stabilization in the rate of phylogenetic succession during the nursing period (REML Weighted: p=.038).

As reflected in the lack of statistically significant differences in diversity metrices, only minor microbial signatures associated with emotionality were revealed. Among human infants, *Lactobacillus* and *Bifidobacterium* strains known to populate the infant gut in response to

breastfeeding, such as *B. longum* and *L. reuteri*, have been demonstrated to possess psychotropic effects, and have been reported to be anxiolytic [32,74]. However, there were no effects of emotionality observed on the rate of acquisition of milk oligosaccharide-consuming commensal taxa. A secondary exploratory analysis was then conducted on other developmentally-relevant genera, and the successive colonization of the Firmicute *Faecalibacterium* was found to be associated with the HIP profiles more directly associated with emotionality. Among normally developing infants, *Faecalibacterium* is low in abundance during the peak nursing period (on average 2.8% at 2 months postnatal) and increases to 3.8% at around 4-6 months of age, before stabilizing at around 3.3% after the infant is weaned from its mother and placed into peer-housing. Infants demonstrating either the behaviorally reactive or the inhibited temperamental phenotype exhibited atypical *Faecalibacterium* colonization (REML Reactive: p=.024; Inhibited: p=.053), characterized by higher abundances at peak nursing (~4%) and lower abundances (~2%) during postnatal months 8-10 when compared to less emotionally reactive infants (Figures 5A & 5B). This association was no longer apparent by 12 months of age.

From the mother-infant observations, it was hypothesized that these differences in *Faecalibacterium* acquisition result in part from differences in consummatory behavior. Both behaviorally reactive and behaviorally inhibited infants spent less time engaging in behaviors associated with breastfeeding at the peak nursing period, such as mutual contact with the mother's ventrum ($F_{(1,22)}$= 5.49, p=.028 and $F_{(1,22)}$= 6.32, p=.019, respectively). Additionally, breastfeeding-related behaviors during peak nursing appear to have an enduring relationship with infant gut composition. Specifically, more time spent in contact with the mother's nipple

and ventrum at 2 and 3 months of age was predictive of increased abundance of *Faecalibacterium* during the postweaning period (Nipple contact: 2 mos.: (r=.44, p=.047), 3 mos.: (r=.39, p=.072); Ventrum contact: 2 mos.: (r=.41, p=.056), 3 mos.: (r=.46, p=.026)). These patterns suggest that emotionally reactive infants are transitioning to solids at an earlier age, and that early life dietary patterns may be reflective of more sustained infant temperamental phenotypes.

After evaluating the microbial succession, temperament phenotypes were evaluated in conjunction with microbiome indices that were sampled more closely to the administration of the HIP test. The establishment of a more emotionally reactive temperament was hypothesized to be associated with diminished bacterial diversity in the infant gut after the infant was weaned from the mother. Though not present in samples collected at 8 and 10 months of infant age, the gut microbiota of inhibited infants exhibited reduced richness at 12 months ($F_{(1,22)}$= 5.51, p=.028). However, analysis of weighted (phylogenetic and abundance-based) Unifrac and Jaccard (presence-absence) distance matrices indicated that an effect of infant emotionality did not extend to variation in gut community structure. When evaluating the abundances of oligosaccharide-consuming commensal taxa, there was again no association with the more direct measures of infant emotional reactivity. *Lactobacillus* abundances were, however, found to be diminished in the guts of inactive infants (15% vs. 22%, $F_{(1,22)}$= 4.03, p=.057). Though not statistically significant, these results are in agreement with previous findings from the mother-infant observations that more active and autonomous infants host higher levels of *Lactobacillus*. Finally, contrary to expectation, there was no bacterial correspondence between activity levels and emotionality at any time point, which suggests that

measures of inactivity in response to a novel environment are not independently and uniquely predictive of behavioral inhibition.

ANALYSIS 1: CONCLUSIONS

Results 1.1: While infant behavioral dispositions were generally consistent over time, there was only a trending effect of infant temperament on the rate of the diversification of the infant gut. Emotionally reactive temperaments were not predictive of diminished acquisition of *Lactobacillus* and *Bifidobacterium* but were associated with an earlier transition to solid foods and alterations in the commensal taxa *Faecalibacterium*.

Results 1.2: Dietary transitions were not reflected in changes in the microbial community structure but did correspond to alterations in the prevalence of several major taxa. Levels of *Bifidobacterium* were predictive of behaviors associated with breastfeeding, however, *Lactobacillus* abundances corresponded to the transition to solid foods. There was no observable influence of maternal caregiving behavior on the infant microbiome.

Results 1.3: At 2 weeks of age, poor emotional regulation was predictive of accelerated acquisition of a phylogenetically diverse and abundant microbiota and lower abundances of *Bifidobacterium*, and during 2-4 months of infant age, higher abundances of *Lactobacillus* were observed in the guts of more autonomous infants. Microbial evenness was not predictive of infant emotionality or the adoption of independence at any timepoint.

Analysis 2: Neurodevelopment & Gut Microbial Trajectory

The primate brain and the microbial community structure are maturing concurrently, and disruption of the microbial succession within the infant gut has been previously associated with increased risk for neurodevelopmental disorders [75]. While much is still unknown regarding the exact mechanisms through which the gut microbiota are altering brain development, rodent models have demonstrated that neurodevelopment in the absence of bacteria dramatically affects CNS circuitry, and deficits have been specifically documented in neural regions involved in emotionality, such as the hippocampus, amygdala, and prefrontal cortex [18–21]. Given that brain structure can be associated with brain function [76,77], it was proposed that the temperament phenotypes identified in our prior analyses could be reflected in structural differences in the brain, and furthermore, that variations in the succession of the gut microbiota may correspond to neuroanatomical maturation during the first year of life in the infant monkey.

To this end, the rhesus infants that were included in the behavioral assessments for Analysis 1 underwent magnetic resonance imaging scans at 12 months of age to quantify the structural volumes of total brain and major cortical regions. In addition to the homology with humans in the anatomy of neural circuits mediating stress and social behavior, non-human primates demonstrate an extended period of neurodevelopment postnatally. Within the rhesus monkey brain, maturational changes in total gray matter continue until 2 years of age (with the exception of the prefrontal cortex), whereas total white matter increases throughout the prepubertal period between 2-4 years of age, before plateauing in young adulthood [78]. This extensive period of postnatal maturation provides an extended developmental window during

which the gut bacteria can influence the programming of central neural circuitry associated with emotionality and stress responsiveness [79].

In the current analysis, total brain volume was measured and a regional parcellation template and segmentation were utilized to quantify the size of distinct neuroanatomical regions and structures. The primary analyses focused on the volumes of the amygdala and hippocampus as these regions have a critical role in regulating socioemotional behaviors, and manipulation of the gut microbiota of rodent models has been found to alter the structure and neurochemistry of these regions [18–21]. Secondary analyses focused on tissue volume, as well as the segmentation of white and gray matter in additional parcellated brain regions associated with emotionality and emotion regulation, including the insular and prefrontal cortices, cingulate, cerebellum, and the temporal limbic cortex. In humans, adults with affective and psychotic disorders exhibit higher reactivity to stressful stimuli and have been found to have reduced gray and white matter volumes in the hippocampal, cingulate, and prefrontal cortices [80]. These areas have also been shown in studies of non-human primates to be important for regulating behavioral and physiological reactivity to aversive stimuli [33,81]. Conversely, hyperactivity and expansions in volume of the amygdala have been reported in both inhibited humans and non-human primates [82,83]. Therefore, the following results were expected:

ANALYSIS 2: HYPOTHESES & PREDICTED RESULTS

Hypothesis 2.1: Infant temperament phenotypes will correspond to structural differences in brain regions associated with emotionality.

Predicted Findings 2.1: Emotionally reactive infants are expected to have reduced gray matter volumes and myelination in specific limbic and cortical areas, such as the hippocampus and the prefrontal and cingulate cortices. Conversely, because amygdala hyperactivity has been associated with early life stress, animals with a temperamental disposition to be behaviorally inhibited are expected to have larger amygdala volumes.

Hypothesis 2.2: Individuals demonstrating more advanced neural maturation are predicted to host more mature microbiomes during critical windows of development.

Predicted Findings 2.2: Infants with larger volumes of total gray and white matter within the brain are expected to host a more even microbiome, characterized by higher abundances of bacteria known to facilitate energy extraction from food and host metabolism, such as Firmicutes [84].

Hypothesis 2.3: Certain structural differences in brain areas related to infant temperament will be associated with abnormal microbial succession during the first year of life.

Predicted Findings 2.3: Infants with emotionally reactive temperaments will have atypical gut microbial colonization, specifically with regard to the acquisition of microbial richness and diversity, as well as diminished post-weaning abundances of *Faecalibacterium*.

ANALYSIS 2: METHODS

Brain Scan Procedures and Magnetic Resonance Methods. The magnetic resonance imaging (MRI) scans were obtained with sedation between 12-14 months of age. The monkeys received

an initial dose of ketamine (10-15 mg/kg IM), a dissociative anesthetic with amnestic properties, followed by dexdomitor (0.015 mg/kg IM) for immobilization and sedation during the scan. Plane of anesthesia was monitored with a pulse oximeter, and body temperature maintained during the scan. Thus, the monkeys were not conscious of the scanning procedures, and the immobilization did not elicit distress or discomfort.

The magnetic resonance methods have been previously described by Short et al. (2010)[85]. Briefly, imaging was performed on a GE MR750 3.0T scanner (Milwaukee, Wisconsin) using the human 8-channel brain array coil at the Waisman Laboratory for Brain Imaging and Behavior. A structural atlas that was previously created using juvenile rhesus brains served as the standardized template for determining estimated tissue maps, lobar parcellation, and subcortical structures [78]. The atlas was applied to each infant's structural image in order to create probabilistic tissue maps, which were employed for T1- and T2-based tissue segmentation of cerebrospinal fluid (CFS), gray matter (GM), and white matter (WM). Deformable image registration was used to calculate subcortical structure segmentations. Tissue, lobar region, and subcortical structure volumes were extracted from the segmentation. Analyses of acquired data included cortical regions involved in emotional processing, namely the prefrontal cortex, the temporal limbic region, the cerebellum, and the cingulate and insular cortexes. Within the limbic system, the subcortical volumes of the amygdala and hippocampus were also evaluated.

ANALYSIS 2: RESULTS AND DISCUSSION

Covariate Determination. On average, there was some sexual dimorphism in brain structure, requiring the inclusion of the monkey's sex as a covariate. Total brain volume (TBV) was

calculated by combining total gray matter (GM) and white matter (WM) as defined by the automatic tissue segmentation process. Like male humans [86], male macaques have a significantly larger TBV at 12 months of age ($F_{(1,22)}$= 4.34, p=.045). Male brains were on average 1.06% larger than those seen in females. When accounting for TBV, males also had larger volumes in several regions associated with emotionality, including both gray and white matter within the right insular cortices (GM: $F_{(1,21)}$= 4.46, p=.046 and WM: $F_{(1,21)}$= 7.33, p=.013) and in the left amygdala, although this effect failed to reach significance (p=.075). In order to account for variance explained by sex differences in brain mass, TBV was included as a covariate in the following analyses of brain regions. In general, there was not a significant hemispheric asymmetry in these volumetric measures requiring an additional analysis of the right and left sides of the brain.

Structural Variation in the Brain of Reactive Temperament Phenotypes. After establishing the presence of sex differences in brain size, it was investigated whether the temperament phenotypes previously identified with the HIP, were predictive of structural differences in brain morphology. When evaluating the total overall volumes of white and gray matter, infant temperament phenotype was not associated with this aspect of brain development. Additionally, when controlling for TBV, bihemispheric structural variation was not correlated with the infant levels of motoric activity. There were, however, several differences in affect-associated brain regions in the behaviorally reactive and inhibited infants (Table 2). Among behaviorally reactive infants, there was, as expected, a trend toward smaller volumes in both the left and right hippocampus (Left: $F_{(1,21)}$= 4.13, p=.056; Right: $F_{(1,21)}$= 3.89, p=.069)). This

effect of behavioral reactivity on hippocampal volume was also apparent when considering the earliest measures obtained with the IBAS testing at 2 weeks of age: poorer state control was associated with a smaller volume of the right hippocampus (Right: r= .41, p=.058). Volumetric differences in the hippocampus were not observed for the infants with behaviorally inhibited temperaments. However, they did exhibit smaller GM volumes within the cingulate cortex (Left: $F_{(1,21)}$= 7.64, p=.011; Right: $F_{(1,21)}$= 7.80, p=.01)).

The cingulate cortices are involved in emotion processing and regulation and have direct projections to the motor cortex and to regions within the spinal cord involved in motor activity [87]. Previous studies in rhesus monkeys have demonstrated that the ability to regulate freezing behavior corresponds to activation of regions within the dorsal anterior cingulate cortex [73]. Amygdala morphology has also been reported to differ in individuals with an emotionally reactive temperament [82,83]; however, there were no structural differences in the amygdala of either behaviorally reactive or inhibited infants when compared to less reactive infants (Table 2). It is possible that the effects of early life condition on amygdala volume may not become more evident until an older age, specifically after puberty, which occurs at 3-4 years of age in monkeys [88].

Interestingly, structural differences in the cerebellar regions of the brain were found in both behaviorally reactive and inhibited infants. While the cerebellum has traditionally been associated with motor functioning and planning, more recently attention has been given to its involvement in affect, cognitive processes, and emotion regulation [89,90]. Within infant monkeys, cerebellar WM volume was significantly larger in inhibited animals in both hemispheres of the cerebellum (Left: $F_{(1,21)}$= 8.66, p=.008; Right: $F_{(1,21)}$= 11.00, p=.003)), and

the volume of cerebellar GM was increased in reactive infants (Left: $F_{(1,21)}$= 7.57, p=.012; Right: $F_{(1,21)}$= 4.72, p=.041)). Within humans, differences in cerebellar volumes have been described in certain neurodevelopmental disorders and the age of the child appears to influence whether a larger or smaller volume is reported. For example, hyperplasia of cerebellar WM was reported to occur in early life (at ages 2-3 years) in children with autism spectrum disorder [91]. This early overgrowth was followed by an atypical delay in growth, resulting in reductions in both gray and white matter observed in the adolescent cerebellum. Within infant monkeys, it was similarly observed that infants who scored higher on measures of sensory sensitivity at 2 weeks of age, as determined through their reaction to vestibular and tactile stimulation, also exhibited larger cerebellar GM in the right hemisphere and increased WM in both right and left hemispheres at one year of age (GM Right: r= .42, p=.065 & WM Right: r= .44, p=.051; WM Left: r= .47, p=.04). Excessive and atypical responses to sensory stimulation in infancy have been associated with the emergence of a socially withdrawn and anxious temperament and are a well-documented characteristic of autism spectrum disorder [40,45].

Association Between Gut Microbiome and Brain Development. The next phase of the analysis determined whether microbial succession in the infant gut across the first year of life was predictive of overall brain volume, as well as the volume of structural regions identified as associated with infant temperament. In Chapter 2 it was shown that more phylogenetic diversity and microbial richness from 2 to 6 months of age was predictive of faster and larger weight gains. Infant growth trajectories were predictive of larger total brain volume at one year of age (r=.49, p=.014). However, more gut microbial diversity during the first 6 months of life

did not correspond to larger neural volumes at one year of age. Instead, a larger total brain volume was associated with more evenness of microbial taxa within the gut at 2 months of age (r=.50, p=.011). This finding would concur with the relationship between the patterns of microbial colonization and linear skeletal growth (rate of change in crown-rump length) and also the infants' weight gain trajectories. The volume of GM also appeared to reflect a similar relationship to the one found between skeletal growth and the timing of the gut microbial succession. The abundance of Firmicutes at 2 months of age was associated with larger volumes of GM (r=.39, p=.052). Conversely, the opposite relationship was seen with respect to the abundance of Proteobacteria (r=-.44, p=.027; Figure 6). A higher abundance of Firmicutes has been reported to be a microbial signature of more efficient host metabolism [84]. Within the context of this study, it was also reported that their abundance accompanies the dietary transition from breast milk to solid foods (a correlation reported when discussing the infants' consummatory behavior during the mother-infant observations). Conversely, Proteobacteria dominate the gut microbiota of the younger, nursing infant. WM volumes were not found to be uniquely associated with any individual taxa. This lack of association may be due to temporal differences in the maturational trajectory of GM and WM, and the process of myelination continuing for longer until the pubertal transition [78].

After establishing that some features of brain development did appear to be sensitive to certain aspects of gut microbial composition, it was investigated whether microbial succession was predictive of the growth and volumes of regions identified as being associated with infant temperament. Some overlap was observed between the patterns of microbial community structure found to be associated with emotional reactivity, however, these effects were small

and no longer statistically significant after FDR correction. For instance, GM volumes in the cerebellum, which were larger in behaviorally reactive infants, were only nominally associated with more microbial richness and phylogenetic diversity in the weaned monkey (8-10 months of age). However, this association did not survive statistical correction for multiple comparisons. When evaluating specific taxa, the abundances of *Faecalibacterium* in infants after weaning to solid foods (8-10 months of age) were negatively associated with the volume of both GM and WM in the cerebellum, but the association attained statistical significance in only one side of the cerebellum (GM Left: r= -.39, p=.07 & GM Right: r= -.47, p=.025; WM Left: r= -.50, p=.048 & WM Right: r= -.42, p=.06). The relationships between microbial community structure and abundance with hippocampal or cingulate cortex volume did not retain statistical significance after correcting for multiple comparisons. Finally, it was not possible to demonstrate that the temperament phenotype of the infant mediated any of the observed relationships between bacterial taxa and brain size at one year of age.

ANALYSIS 2: CONCLUSIONS

Results 2.1: Emotionally reactive infants exhibited a smaller volume in limbic structures and cortical regions, including the hippocampal and cingulate cortices, as well as a larger volume of both GM and WM in the cerebellum.

Results 2.2: Infants with larger total brain GM and WM volumes had a more even microbiome during the peak period of nursing, characterized by a higher abundance of Firmicutes.

Results 2.3: There was not a significant mediating influence of bacterial colonization on the relationship between infant temperament and brain development. Postweaning abundance of

Faecalibacterium in the infant gut was negatively associated with GM and WM volumes in the cerebellum, but the effect retained statistical significance on only one side of the cerebellum.

GENERAL DISCUSSION

In this observational study, the correspondence between the maturation of gut microbiome community and brain and behavioral development was investigated. The results showed that the relative abundances of several commensal taxa were affected by consummatory behavior and were associated with the infant activity levels, including the abundances of *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium*. Atypical acquisition of *Faecalibacterium* was also associated with a more emotionally reactive infant temperament, and at 12 months of age, was predictive of differences in the volume of several neural structures and regions associated with emotion regulation. A delayed acquisition of bacterial community evenness was additionally predictive of smaller total brain volume. Although there were few results that retained statistical significance after correcting for multiple comparison, our findings contribute to the extant literature suggesting that early life experiences, such as infant diet and environmental exploration, are associated with the composition and community structure of the gut microbiome.

While a specific influence of maternal caregiving and solicitousness on the infants' microbiota was not detected, the more overt maternal contributions through breastfeeding were clearly evident. As hypothesized, a higher abundance of the commensal taxa *Bifidobacterium* was associated with suckling behavior indicative of nursing and the consumption of breast milk, as well as nonnutritive contact with the nipple. Following the

patterns described for human infants, active nursing and a predominance of *Bifidobacterium* within the infant gut were also predicted to be associated with lower gut microbial diversity [92]. However, the developmental shifts in microbial richness and diversity were apparent only as infant nutrition became more varied and transitioned to include solid foods during the latter stages of nursing. In the infant monkey, the abundances of the typically beneficial taxa, *Lactobacillus*, were also increased at an older age when the infant transitioned to solid foods. This microbial sequence followed the patterns of colonization described previously in Chapter 2, where *Lactobacillus* was found to increase most dramatically following weaning from the mother, when the infant shifted to exclusively consuming the fiber-rich monkey biscuits. Conversely, in vaginally-born and breastfed human infants, *Lactobacillus* is present at higher abundances from the time of delivery and increases dramatically over the first 6 weeks of life [68,71]. The distinctive microbial succession observed in the non-human primate infant gut suggests that there may be differences in the composition and prebiotic properties of breast milk in certain primate species. Future studies are now needed to identify and quantify the concentrations of the oligosaccharides in rhesus milk to determine the prebiotic potential to promote the growth of these commensal strains.

A consistent association between infant emotional reactivity and *Bifidobacterium* and *Lactobacillus* was also not detected, despite previous reports of lower abundances of these commensal microbes in the guts of irritable human infants with colic and in infant monkeys in response to stressful experiences [34,93,94]. Higher *Lactobacillus* abundances were, however, consistently associated with behaviors associated with infant autonomy and activity during the nursing period between 2-4 months of age. It is possible that the failure to replicate stronger

associations with these commensal taxa may be due to the infant monkeys hosting different species when compared to those typically reported within the gut of the human infant that have been associated with psychotropic effects, such as *B. longum* and *L. reuteri* [32,74]. Human and non-human primate infants appear to be colonized by *Lactobacillus*, both of different species and of different bodily origins. In women of European backgrounds, *Lactobacillus* dominates the conserved vaginal microbiota and infants are colonized by exposure to this lactic acid-producing bacteria largely during delivery [95]. Conversely, the vaginal microbial composition of non-human primates is highly diverse, and *Lactobacilli* abundance is sparse in their reproductive tract [96]. However, members of the genus *Lactobacillus* are among the more abundant taxa in the gut of the adult macaque. Therefore, rhesus infants that display more independence and activity at a younger age may be exposed to more fecal matter and *Lactobacilli* as they explore their physical environments. It also appears that some of the nutrients in the biscuits that are the primary constituent of the monkeys' diet contain prebiotic properties that have been associated with the growth of *Lactobacillus* [97]. Given the potential differences in the species of *Lactobacillus* that predominantly colonize the infant rhesus and infant human, and the possibility of a different source – either at delivery or later exposure to the environment, it will require additional research to further interrogate the reasons for the differences between primate species. Unfortunately, although 16S rRNA gene sequencing is very useful for bacterial classification, it has low power for taxonomic identification at the species level [98]. Future metagenomic studies utilizing deeper gene sequencing are needed to identify commensal microbes at the species and strain level.

Although associations between milk oligosaccharide-responsive microbes and temperament were not observed, emotionally reactive infants were found to exhibit differences in *Faecalibacterium* succession. Specifically, emotionally reactive infants hosted higher relative abundances of *Faecalibacterium* at peak nursing and lower abundances were found following weaning from the mother. These differences in the abundance of *Faecalibacterium* are notable because prior research in humans and rodent models has suggested that the sole species within the genera, *Faecalibacterium prausnitzii,* has the potential for psychoactive effects, with anxiolytic and antidepressant-like actions [24,99]. These effects have been attributed to the production of butyrate, a short chain fatty acid (SCFA) with intra- and extra-intestinal activity, including enhancing the integrity of the gut barrier and reducing inflammatory and oxidative stress [27]. The abundance of *F. prausnitzii* is often found to be lower with intestinal dysbiosis such as found in irritable bowel syndrome (IBS) and colitis [100]. Dysbiotic changes in microbial composition have also been found to occur during periods of psychological stress. In our monkey colony, infants between 6 and 8 months of age are routinely separated from their mothers and rehoused in social groups with 3-4 similarly aged individuals. Previous research has already demonstrated that the disturbance associated with maternal separation evokes a significant cortisol response, and transient decrease in *Bifidobacterium* and *Lactobacilli*, with a concurrent increase in other taxa [34,101]. Although the observational nature of the current analyses precludes a determination of causality, it is possible that infants with a more emotionally reactive temperament experience greater distress upon maternal separation, and subsequently have a reduced abundance of *Faecalibacterium* for a sustained period of time.

Differences observed in the succession of *Faecalibacterium* may additionally indicate that early consummatory behavior has an enduring influence on gut microbial composition that persists beyond the transition from breastmilk to solid foods. Within the current study, the emotionally reactive infants breastfed less frequently during the period of peak nursing. Coupled with higher abundance of *Faecalibacterium* in the gut at 2 months of age, this finding suggests that emotionally reactive animals exclusively breastfed for a shorter duration during infancy. Studies in piglets have reported that differences in diet and rearing husbandry, such as early weaning, may disrupt the development of the gastrointestinal barrier, resulting in increased intestinal permeability and intestinal dysbiosis, and ultimately increased risk of diarrheic morbidity [102,103]. Significantly, in Chapter 2, early colonization by genera within Firmicutes, such as *Faecalibacterium*, was reported to be associated with diminished microbiome richness and delayed maturation of the microbiome community structure. This association suggests that higher relative abundances of *Faecalibacterium* during the nursing period, coupled with a diminished abundance after weaning, may be a microbial signature of a more dysregulated gut that would be associated with inflammation and dysbiosis. Taken together, the findings in infant animals, including monkeys, suggest that early dietary decisions have implications for later digestive health and overall wellbeing. However, more controlled studies that include an experimental manipulation of infant diet are still needed to more definitively demonstrate the extent to which early nutrition influences the maturation of the gut and gut microbiome. The experiment described in the final chapter of this thesis exemplifies that type of empirical interrogation of the importance of breast milk in supporting the healthy microbiome of the young infant.

Our findings also provide further evidence that the infant's physical and later neurodevelopment outcomes may also be sensitive to aspects of the microbiome, such as the evenness of the microbial community distribution within the gut. In Chapter 2, it was reported that evenness within the microbial distribution is acquired early and reaches adult-like levels by 4 months postpartum. Infants displaying a delayed acquisition of a more even and balanced microbiome were found to have smaller total brain volumes at 12 months of age. This relationship is likely associated with our previous finding which showed that evenness within the gut microbiome community was associated with growth, including faster skeletal growth and larger weight gain trajectories (Chapter 2). Beyond just impacting infant growth [104], studies in germ-free mice have indicated that commensal bacteria are independently needed for normal morphological development and maturation of the brain [105].

To our knowledge, this study is the first longitudinal experiment to provide evidence for an association between microbial succession and temperamental phenotypes in rhesus monkeys. There are, however, a number of limitations and factors that should be acknowledged. First, the study design was observational and the hypotheses were exploratory, and therefore, did not allow for determination of causal relationships or a deeper insight into some of the underlying mechanisms, such as potential differences in gut physiology. However, the fact that some associations were still evident in a normally developing and healthy cohort of infant monkeys suggests that interactions between the gut microbiota, growth, and behavior can emerge at an early age. Future studies manipulating the infant's exposure to breastmilk and maternal sources of these microbes are needed to more definitively establish these findings. In addition, while structural MRI was employed to determine differences the morphology of the

brain, it was done only at one age point, and volumetric differences do not necessarily translate to functional differences. Further analysis, employing other data acquisition methods, such as diffusion tensor imaging to visualize the microstructural details of WM tracts, or resting state functional MRI to measure resting state neural activity, is now required to understand the significance of the observed differences in GM and WM volume in several brain areas. Incorporating serial neuroimaging at multiple timepoints across the first year of life would also improve the identification and specificity of when CNS development is affected by the gut microbiota. Finally, future investigations should incorporate a larger sample size in order to consider the possibility of a differential relationship between gut bacteria and brain development in females and males. The extant literature suggests that male infants are at greater risk for a number of neurodevelopmental disorders [106], and early disruptions in gut microbial colonization have been reported in rodents to have effects on the hippocampal serotonergic system that are only observed in male pups [12]. While significant sex differences in the gut microbial succession from birth to one year of age were not detected (Chapter 2), nor major sex differences in infant behavior, it is possible that a sex-associated susceptibility would become apparent only after a stressful event or a controlled manipulation of the gut microbiota.

Despite these limitations, the results provide further evidence of bidirectional signaling between the gut and brain early in life. In Chapter 2, the normative patterns of microbial colonization in the infant rhesus gut were characterized, which served as the foundation for correlational analyses with behavior and brain development. Through these descriptive and observational analyses, the following conclusions were drawn: 1) colonization of the gut

microbiome follows a predictable developmental trajectory in the infant rhesus monkey, 2) maturation of the gut microbial community reflects the combined influence of infant diet and early life experiences, such as the husbandry practice of weaning at 6 months of age, and 3) gut microbial composition is associated with several physical and brain outcomes, including growth rate and the GM and WM volumes in a number of brain regions. Collectively, these findings concur with the view that there is a biological expectancy for the mother to provide a sustained microbial inoculation and to reinforce the early gut colonization through the provision of breast milk. Furthermore, early individual differences related to consummatory behavior and emotional temperament can have a synergistic effect on the microbial succession within the gut, with important implications for developmental health. However, in order to more precisely identify the role of the mother and the critical windows of development, an experimental manipulation of early exposure to maternal sources of microbiota is still needed.

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Chapter 3: FIGURES & TABLES

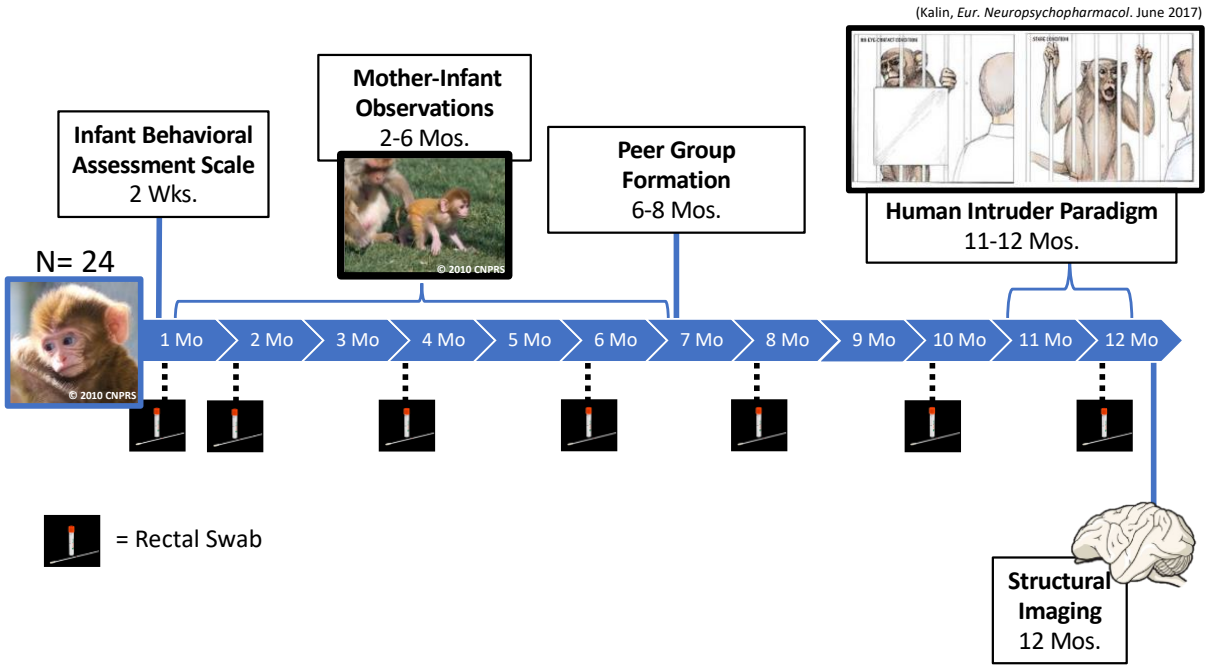


Figure 1. Study Infographic. 24 rhesus monkeys (*Macaca mulatta*) were followed from 2 weeks postnatal to 1 year of age. Standardized assessments of behavior reflective of the development of autonomy, dietary transitions, and emotionality were administered, and rectal swabs were collected at 2-month intervals. The Infant Behavioral Assessment Scale was administered at 2 weeks postnatal and measured emotionality and arousal, attention reactions to visual and auditory cues, responses to vestibular and tactile stimulation, and neuromotor reflexes. From 2-4 months of age, mother-Infant dyad interactions and infant behavior were observed. Attention was given to determinants of infant autonomy, dietary transitions, and maternal solicitousness. Between 10-12 months of age, infants underwent the Human Intruder Paradigm and behavioral displays of emotional reactivity were used to phenotype infant temperament. At 12 months of age, infants underwent structural magnetic resonance imaging and gray matter and white matter volumes were determined for total brain and major cortical regions associated with emotionality.

Figure 2.

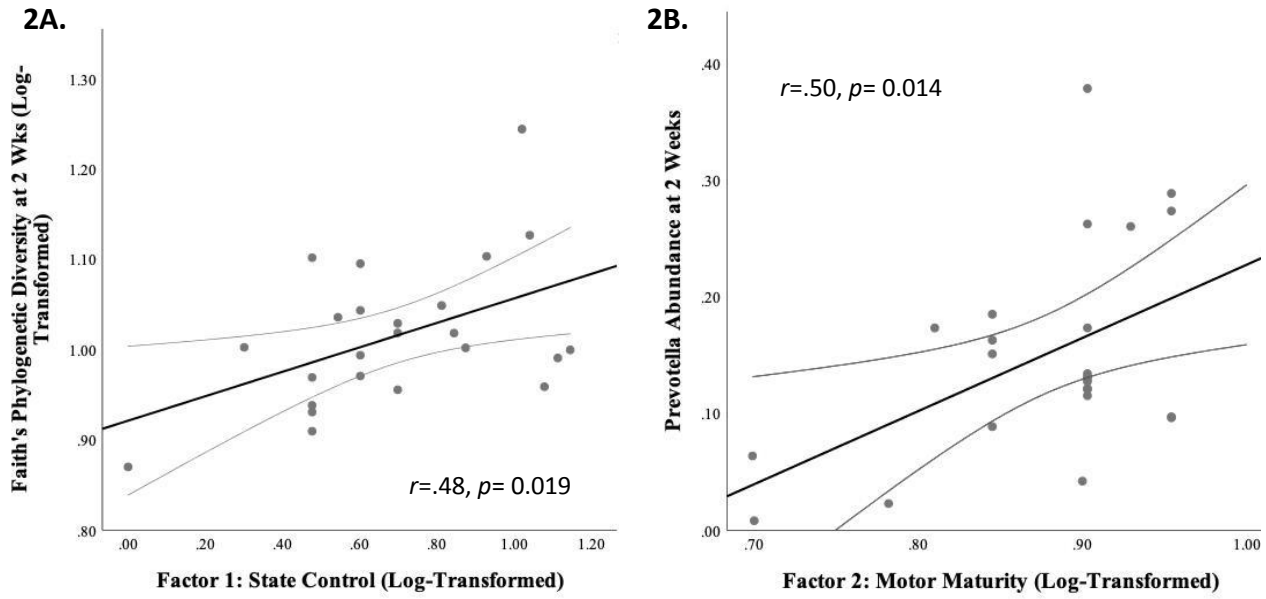
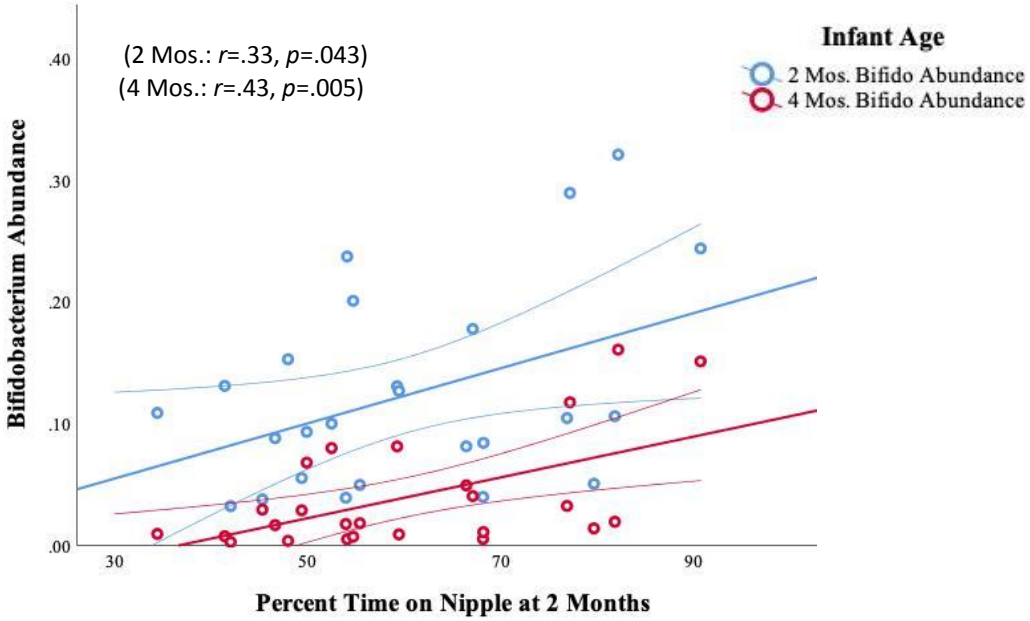


Figure 2. Correlations of Infant Behavioral Assessment Scale (IBAS) composite scores and 2-week microbial abundances with 95% confidence interval error bands. **2A.** Correlation of Faith's Phylogenetic Diversity with the IBAS State Control composite variable at 2 weeks of infant age. Accelerated acquisition of phylogenetic diversity and richness within the infant gut corresponded with poorer emotion regulation in infants. **2B.** Correlation of *Prevotella* abundance with the IBAS Motor Maturity composite variable. At 2 weeks of age, infants that displayed more advanced neuromotor development are host to higher abundances of *Prevotella.*

Figure 3.

3A.



3B.

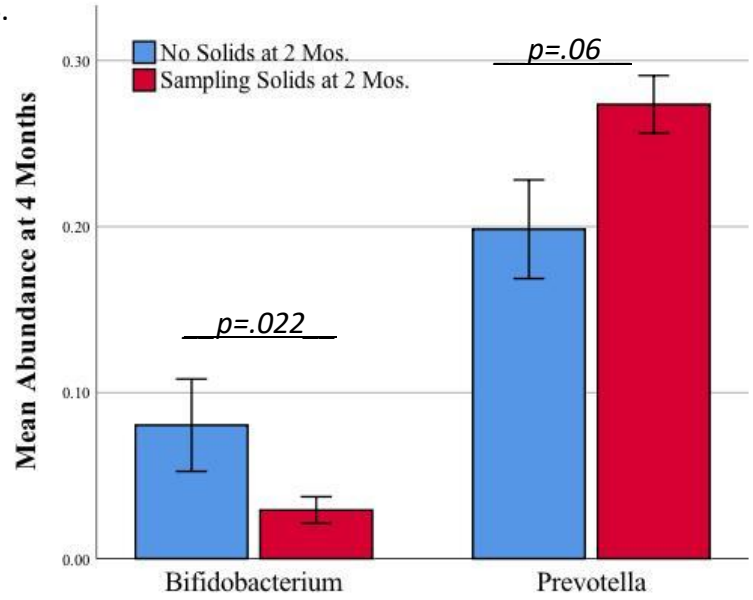
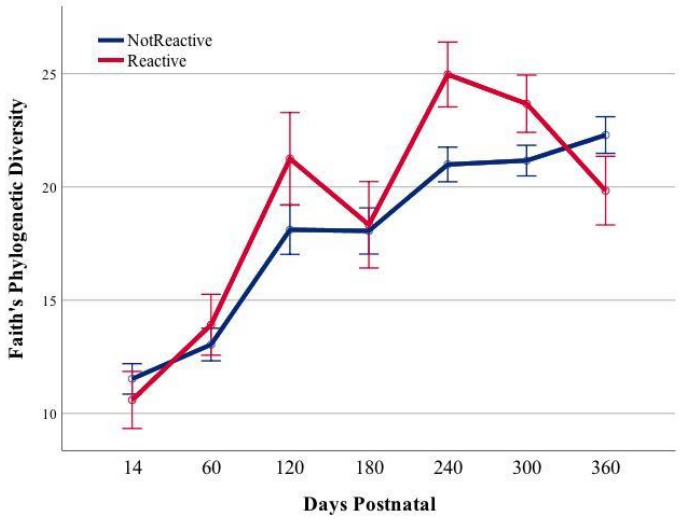


Figure 3. Microbial associations with dietary behaviors recorded during mother-infant observations. **3A.** Association between *Bifidobacterium* and the average percent of time the infant is in contact with the mother's nipple at the 2-month observations. Longer durations spent on the nipple during the peak nursing period were associated with increased abundance of *Bifidobacterium* across the first 4 months of life ($F_{(1,22)}$=6.064, *p*=.022). **3B.** Infants that had begun sampling solids over the 2-month observation period hosted on average lower abundances of *Bifidobacterium* and trended toward higher abundances of *Prevotella* at 4 months of age ($F_{(1,22)}$=3.93, *p*=.06). Bars portray the mean levels (± SEM).

Figure 4.

4A.



4B.

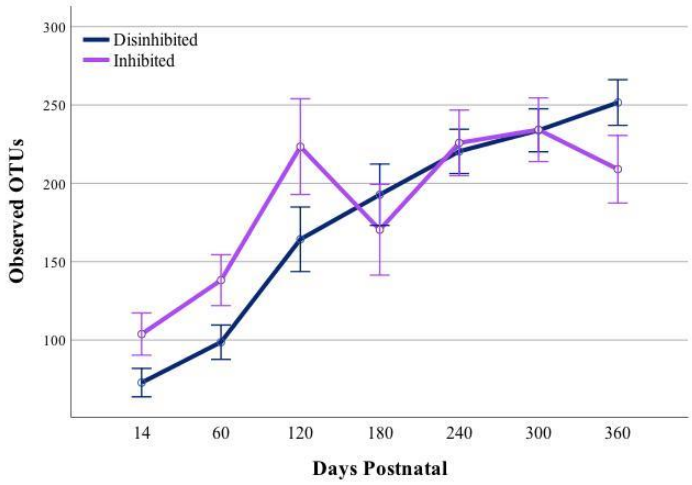


Figure 4. Alpha diversity metrices vary as a function of infant temperament characteristics. **4A.** Behaviorally reactive infants trended toward a less linear acquisition of a more phylogenetically diverse and abundant microbiome (REML Faith's Phylogenetic Diversity: p=.053), characterized by higher phylogenetic diversity following weaning. This effect diminished by 1 year of age. **4B.** Conversely, inhibited infants trended toward hosting higher microbial richness throughout the first 4 months of life (REML Observed OTUs: p=.066). Mean levels (± SEM) are portrayed.

Figure 5.

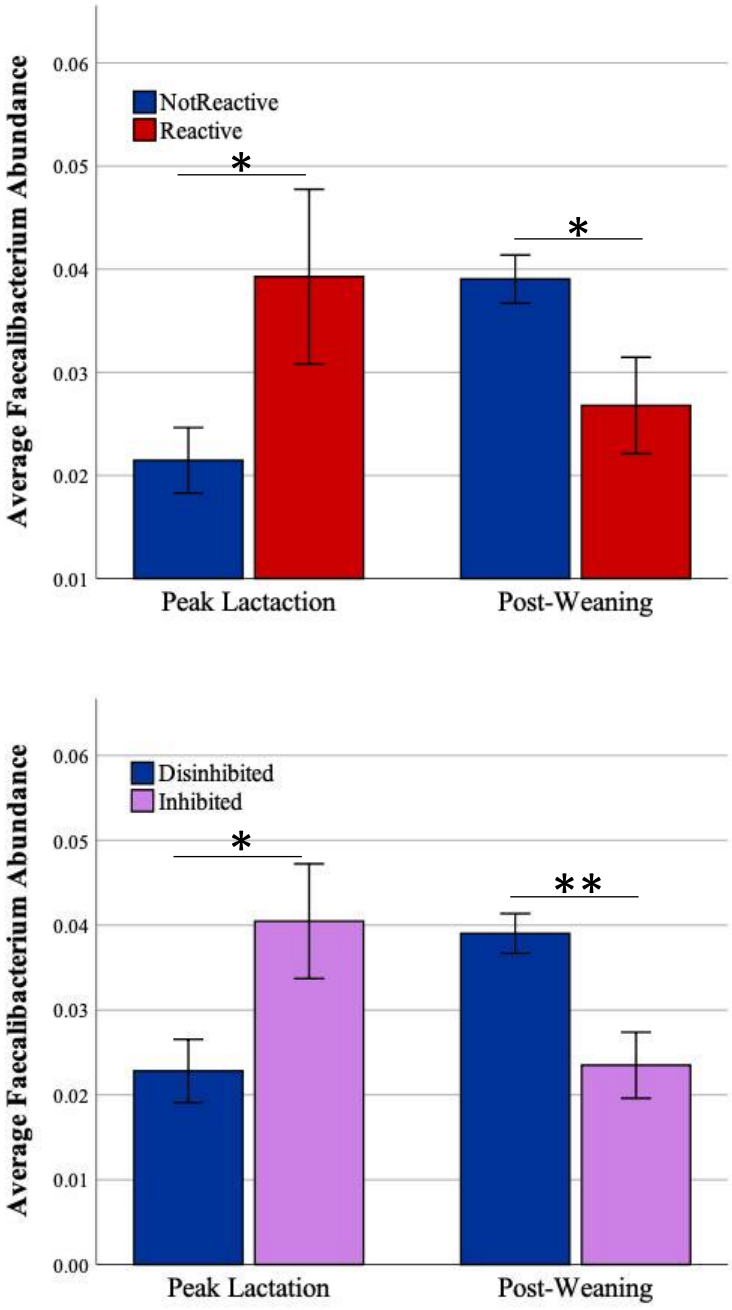


Figure 5. Mean *Faecalibacterium* abundances at peak lactation and post-weaning vary by infant temperament phenotype. Both behaviorally reactive (**5A**) and behaviorally inhibited infants (**5B**) displayed higher *Faecalibacterium* abundances at 2 months postnatal (~4%) and lower abundances (~2%) during postnatal months 8-10, when compared to less emotionally reactive infants. Mean ± SEM values are portrayed. ** p<0.01 difference between temperament phenotypes; * indicates significant difference at p< 0.05.

Figure 6.

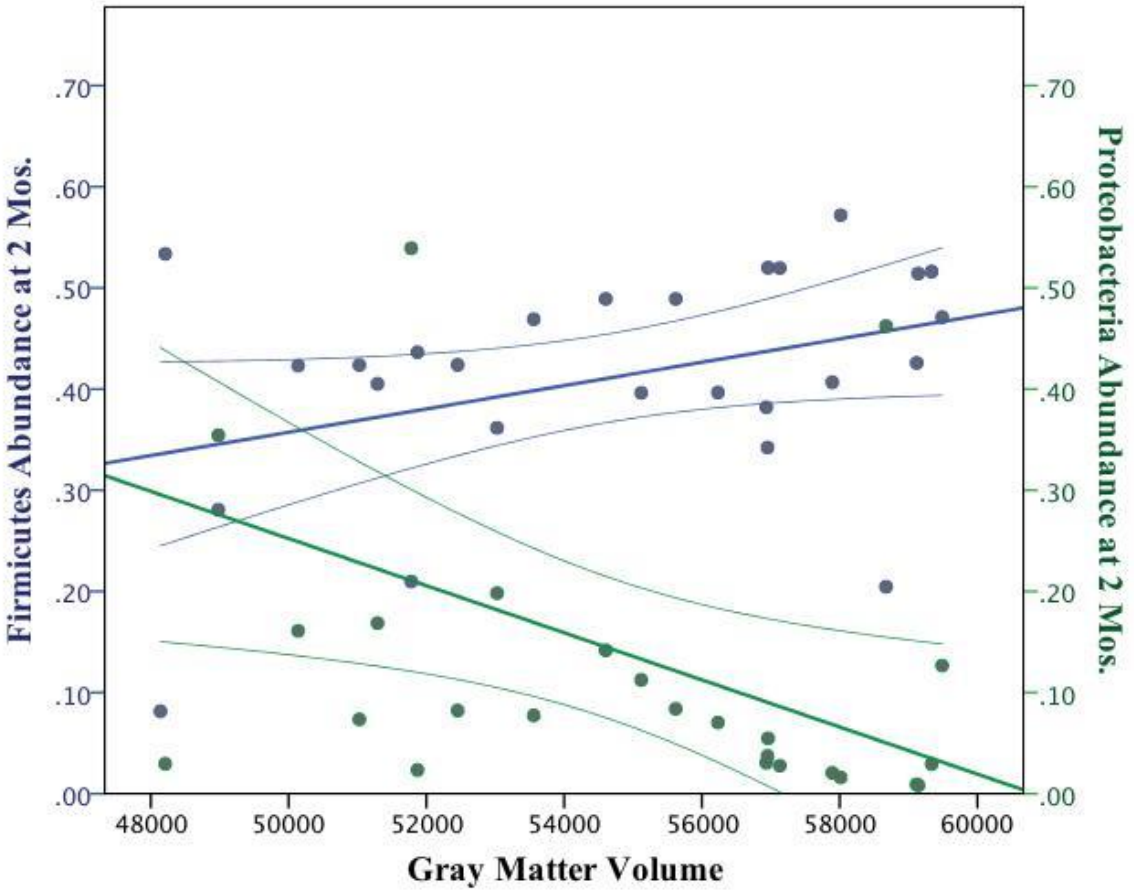


Figure 6. Associations between gray matter volume at 12 months of age and microbial abundances with 95% confidence interval error bands. At 2 months of age, Firmicutes abundance is associated with larger volumes of grey matter (r=.39, p=.052), and an opposing significant relationship is seen with Proteobacteria abundance (r=-.44, p=.027).

Table 2: Brain Volume in Emotion-Associated Regions by HIP Temperament Profile

			Behavioral Inhibition			Behavioral Reactivity		
Subcortical		**Hemisphere**	Disinhibited	Inhibited	***p-value***	Passive	Reactive	***p-value***
Amygdala		Left	306.4 (6.12)	320.32 (12.95)	*NS*	308.1 (6.4)	319.77 (13.99)	*NS*
		Right	313.12 (6.69)	336.01 (12.08)	*NS*	323.13 (6.87)	309.14 (14.53)	*NS*
Hippocampus		Left	358.1 (8.38)	368.63 (13.36)	*NS*	366.73 (7.65)	341.77 (15.09)	*0.056*
		Right	355.74 (7.63)	365.77 (13.52)	*NS*	363.8 (7.02)	340.79 (15.77)	*0.069*
Cortical								
Cerebellum	GM	Left	2601.44 (35.42)	2674.42 (103.68)	*NS*	2572.99 (37.74)	2806.02 (80.68)	*0.012*
		Right	2579.37 (31.96)	2688.19 (112.29)	*NS*	2567.21 (40.58)	2775.48 (89.13)	*0.041*
	WM	Left	967.57 (16.18)	1052.31 (32.82)	*0.008*	990.64 (16.57)	1003.17 (54.01)	*NS*
		Right	959.51 (18.3)	1042.42 (33.8)	*0.003*	974.83 (16.47)	1020.41 (59.43)	*NS*
Cingulate	GM	Left	980.23 (12.03)	916.09 (19.83)	*.011*	969.13 (24.38)	957.93 (37.26)	*NS*
		Right	992.91 (9.29)	942.90 (15.31)	*0.01*	979.97 (24.02)	990.44 (38.2)	*NS*
	WM	Left	188.46 (4.91)	200.71 (11.48)	*NS*	190.5 (5.32)	198.27 (12.54)	*NS*
		Right	180.67 (4.2)	196.99 (10.49)	*0.071*	184.03 (5.01)	191.42 (10.6)	*NS*
Insula	GM	Left	375.47 (10.39)	379.61 (17.31)	*NS*	378.66 (10.69)	369.8 (12.86)	*NS*
		Right	360.32 (8.67)	375.45 (15.52)	*NS*	360.68 (9.14)	380.24 (10.48)	*NS*
	WM	Left	44.58 (2)	46.36 (3.07)	*NS*	46.72 (1.71)	39.35 (3.64)	*0.056*
		Right	41.26 (1.45)	44.59 (1.72)	*NS*	41.26 (1.39)	45.92 (0.45)	*NS*
Prefrontal	GM	Left	2961.42 (71.46)	2975.36 (74.02)	*NS*	2971.01 (59.39)	2946.41 (136.93)	*NS*
		Right	2976.32 (70.07)	2944.92 (66.6)	*NS*	2971.2 (60.22)	2950.79 (113.56)	*NS*
	WM	Left	940.5 (31.03)	991.98 (52.09)	*NS*	944.17 (27.63)	999.37 (74.94)	*NS*
		Right	931.52 (29.42)	987.13 (46.58)	*NS*	933.79 (26.81)	1001.19 (61.75)	*NS*
Frontal Lobe	GM	Left	3246.31 (63.69)	3329.69 (101.4)	*NS*	3269.92 (59.52)	3286.64 (129.24)	*NS*
		Right	3250.83 (66.09)	3292.73 (98.23)	*NS*	3265.75 (60.37)	3261.94 (126.87)	*NS*
	WM	Left	1601.76 (63.2)	1503.18 (36.8)	*NS*	1517.79 (36.76)	1590.77 (73.72)	*NS*
		Right	1500.65 (34.71)	1567.9 (69.45)	*NS*	1517.1 (35.17)	1540.97 (82.13)	*NS*
Temporal Limbic	GM	Left	1267.31 (18.89)	1305.06 (46.91)	*NS*	1276.03 (21.97)	1288.78 (42.8)	*NS*
		Right	1276.73 (20.57)	1324.93 (54.99)	*NS*	1292.57 (24.08)	1287.16 (55.84)	*NS*
	WM	Left	216.04 (3.85)	234.6 (15.78)	*NS*	219.61 (6.54)	229.18 (10.62)	*NS*
		Right	220.5 (3.95)	231.82 (11.61)	*NS*	223.85 (5.34)	224.26 (8.05)	*NS*

Descriptives table for Temperament & Neurodevelopment. Mean Volume (Standard Error). Red indicates decreased volume; Blue indicates increased volume. Trending differences less than p= 0.07 are denoted in lighter shades. Abbreviations as follows: Gray Matter: GM, White Matter: WM.

CHAPTER 3: SUPPLEMENTAL MATERIALS

Supplemental Table 1. Complete Infant Behavioral Assessment Scale and Microbial Correlations

IBAS Factors	**Evenness**	**Faith's Phylogenetic Diversity**	**Observed OTUs**	***Prevotella***	***Lacto.***	***Strept.***	***Bifido.***	***Bacteroides***	***Faecal.***	***Flexispira***	***Caten.***
1. State Control	-0.007	**0.48***	0.274	0.237	-0.021	0.238	-0.213	0.102	-0.037	-0.078	-0.278
	0.969	***0.019***	*0.105*	*0.157*	*0.904*	*0.156*	*0.205*	*0.548*	*0.828*	*0.718*	*0.189*
2. Motor Control	**0.308**	0.038	0.042	**.517***	0.126	0.217	**.338***	**-.561****	-0.155	0.152	0.308
	0.067	*0.826*	*0.806*	***0.01***	*0.457*	*0.197*	***0.041***	***0***	*0.359*	*0.478*	*0.143*
3. Orientation	-0.105	-0.2	-0.195	-0.131	0.157	0.11	0.132	0.056	-0.277	-0.058	0.021
	0.543	*0.241*	*0.253*	*0.441*	*0.354*	*0.517*	*0.438*	*0.74*	*0.097*	*0.787*	*0.923*
4. Sensory Sensitivity	0.225	0.185	0.177	-0.006	0.081	**0.288**	-0.019	-0.25	0.029	0.053	-0.012
	0.187	*0.279*	*0.302*	*0.974*	*0.634*	***0.08***	*0.912*	*0.136*	*0.867*	*0.805*	*0.955*
IBAS Components											
Startle to Auditory	0.209	-0.177	-0.153	**.333***	0.029	0.017	0.029	**-.366***	0.032	0.081	-0.107
	0.221	*0.302*	*0.372*	***0.044***	*0.863*	*0.921*	*0.864*	***0.026***	*0.852*	*0.706*	*0.619*
Response Intensity	0.118	0.23	0.18	0.199	0.012	**0.412***	**-.328***	0.047	0.095	-0.003	-0.318
	0.491	*0.177*	*0.293*	*0.239*	*0.944*	***0.031***	***0.048***	*0.782*	*0.574*	*0.987*	*0.13*
Predominant State	0.03	0.122	0.089	0.178	-0.067	0.134	0.009	0.047	0.064	-0.079	-0.108
	0.864	*0.48*	*0.604*	*0.293*	*0.694*	*0.431*	*0.959*	*0.784*	*0.705*	*0.714*	*0.617*
Vocalizations	0.046	0.21	0.149	0.177	-0.05	**0.327***	-0.194	-0.01	-0.048	0.012	-0.067
	0.792	*0.22*	*0.385*	*0.295*	*0.771*	***0.047***	*0.251*	*0.954*	*0.779*	*0.956*	*0.756*

IBAS Cont.	Evenness	Faith's Phylogenetic Diversity	Observed OTUs	Prevotella	Lacto.	Strept.	Bifido.	Bacteroides	Faecal.	Flexispira	Cateni.
Irritability	-0.032	0.265	0.226	0.196	-0.037	0.156	-0.07	-0.038	-0.027	0.104	0.319
	0.853	*0.119*	*0.185*	*0.244*	*0.828*	*0.355*	*0.682*	*0.823*	*0.872*	*0.63*	*0.129*
Consolability	0.068	-0.202	-0.151	**.380***	0.016	0.158	-0.159	0.128	-0.043	-0.139	-0.359
	0.691	*0.236*	*0.38*	***0.02***	*0.927*	*0.349*	*0.346*	*0.451*	*0.799*	*0.517*	*0.085*
Struggle	-0.03	**.337***	**0.329**	0.192	0.122	**0.312**	-0.108	0.047	-0.234	-0.299	-0.308
	0.864	***0.044***	***0.05***	*0.256*	*0.47*	***0.06***	*0.526*	*0.784*	*0.163*	*0.155*	*0.143*
Fearfulness	-0.024	0.249	**0.314**	-0.28	0.251	-0.055	**-0.378**	**.358***	0.236	0.172	-0.23
	0.89	*0.144*	***0.063***	*0.094*	*0.133*	*0.744*	***0.019***	***0.03***	*0.159*	*0.421*	*0.279*
Soothability	-0.007	**-.404***	**-.364***	-0.122	0.144	-0.202	0.221	-0.168	-0.032	0.117	-0.099
	0.969	***0.014***	***0.029***	*0.471*	*0.396*	*0.23*	*0.189*	*0.321*	*0.849*	*0.587*	*0.644*
Visual Orient	-0.087	-0.098	-0.073	-0.112	0.178	0.087	0.037	0.117	-0.203	-0.096	-0.11
	0.613	*0.569*	*0.671*	*0.507*	*0.292*	*0.608*	*0.83*	*0.492*	*0.256*	*0.654*	*0.608*
Visual Follow	-0.147	-0.043	-0.028	-0.153	0.209	0.13	0.124	0.148	-0.125	0.333	0.362
	0.391	*0.801*	*0.87*	*0.367*	*0.214*	*0.443*	*0.466*	*0.381*	*0.39*	*0.112*	*0.082*
Orient to Auditory	0.123	-0.075	-0.095	0.259	-0.018	0.065	**0.321**	**-.361***	-0.223	-0.146	-0.055
	0.475	*0.663*	*0.583*	*0.122*	*0.917*	*0.704*	***0.053***	***0.028***	*0.184*	*0.497*	*0.797*
Duration of Looking	-0.099	-0.18	-0.179	-0.135	0.175	0.036	0.062	0.129	-0.275	-0.096	-0.11
	0.566	*0.294*	*0.296*	*0.424*	*0.301*	*0.83*	*0.716*	*0.447*	*0.125*	*0.654*	*0.608*
Distractible	0.048	0.29	0.284	0.144	0.095	0.037	-0.222	0.181	0.035	-0.008	-0.26
	0.783	*0.086*	*0.094*	*0.395*	*0.576*	*0.828*	*0.187*	*0.283*	*0.837*	*0.969*	*0.219*
Attention	-0.093	-0.217	-0.219	-0.225	0.226	0.214	-0.047	0.067	-0.164	0.04	0.122
	0.589	*0.203*	*0.199*	*0.181*	*0.179*	*0.203*	*0.784*	*0.695*	*0.333*	*0.852*	*0.571*
Response speed	0.028	-0.107	-0.139	0.232	-0.022	0.066	0.226	-0.074	-0.005	-0.009	-0.087
	0.873	*0.534*	*0.419*	*0.168*	*0.897*	*0.697*	*0.178*	*0.665*	*0.978*	*0.968*	*0.687*

IBAS Cont.	Evenness	Faith's Phylogenetic Diversity	Observed OTUs	*Prevotella*	*Lacto.*	*Strept.*	*Bifido.*	*Bacteroides*	*Faecal.*	*Flexispira*	*Cateni.*
Coordination	0.223	0.116	0.09	**.431****	0.072	0.302	**.372***	**-.449****	-0.152	0.2	**.472***
	0.192	*0.499*	*0.602*	***0.008***	*0.673*	*0.069*	***0.023***	***0.005***	*0.369*	*0.349*	***0.02***
Motor Activity	0.135	0.13	0.084	**.448****	0.169	0.153	0.079	**-0.384***	-0.091	0.033	-0.061
	0.431	*0.451*	*0.625*	***0.005***	*0.318*	*0.367*	*0.641*	***0.028***	*0.592*	*0.878*	*0.777*
Passive	-0.16	-0.213	-0.208	-0.167	-0.21	-0.255	0.154	0.097	0.238	-0.103	-0.25
	0.35	*0.213*	*0.225*	*0.324*	*0.212*	*0.128*	*0.363*	*0.567*	*0.156*	*0.631*	*0.239*
Restrain	-0.022	0.286	0.256	**.398***	0.051	0.18	-0.18	0.138	-0.013	-0.215	-0.114
	0.901	*0.091*	*0.131*	***0.015***	*0.763*	*0.288*	*0.285*	*0.415*	*0.938*	*0.313*	*0.596*
Cuddliness	0.111	-0.252	-0.135	-0.223	0.039	-0.224	0.159	-0.088	0.041	0.141	0.323
	0.519	*0.138*	*0.432*	*0.184*	*0.819*	*0.182*	*0.348*	*0.603*	*0.809*	*0.511*	*0.124*
Calming self	0.052	-0.258	-0.204	-0.178	0.002	-0.186	**.371***	-0.178	0.097	0.027	-0.18
	0.762	*0.128*	*0.232*	*0.293*	*0.991*	*0.269*	***0.024***	*0.293*	*0.568*	*0.9*	*0.4*
Tactile Response	0.129	0.015	0.083	-0.111	0.121	0.084	**-0.301**	0.038	-0.082	**.459***	0.055
	0.455	*0.932*	*0.631*	*0.514*	*0.475*	*0.622*	***0.065***	*0.824*	*0.629*	***0.024***	*0.8*

Correlation table for Infant Behavioral Assessment and abundant genera. Highlighted color identifies IBAS component identification. Spearman-rank correlation coefficients (*p* value provided underneath in italics). Abbreviations as follows: Lactobacillus: Lacto., Streptococcus: Strep., Bifidobacterium: Bifido., Faecalibacterium: Faecal., Catenibacterium: Cateni. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Supplemental Table 2A. Alpha Diversity Metrics and Mother-Infant Observations

		Evenness		FPD		Observed OTUs	
	D/F	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo
Infant Dietary Behaviors							
Eating: 2 Mos.	D	0.215	-0.277	0.43*	0.31*	0.278	0.302
		0.195	*0.083*	*0.015*	*0.047*	*0.091*	*0.062*
Eating: 3 Mos.	D	0.305	-0.028	0.308	-0.138	0.258	-0.146
		0.063	*0.862*	*0.06*	*0.395*	*0.117*	*0.369*
Eating: 4 Mos.	D	0.317	0.17	.364*	-0.199	0.163	-0.189
		0.053	*0.294*	*0.025*	*0.219*	*0.329*	*0.244*
Ventral Contact: 2 Mos.	D	0.105	0.103	-0.222	-0.159	-0.093	-0.189
		0.531	*0.528*	*0.181*	*0.327*	*0.579*	*0.244*
Ventral Contact: 3 Mos.	D	-0.099	0.193	-0.306	0.049	-0.17	0.035
		0.553	*0.234*	*0.062*	*0.762*	*0.307*	*0.83*
Ventral Contact: 4 Mos.	D	-.331*	-0.091	-0.216	0.111	-0.001	0.054
		0.042	*0.578*	*0.192*	*0.496*	*0.995*	*0.74*
Nipple Contact: 2 Mos.	D	0.215	0.088	-0.093	-0.109	0.013	-0.106
		0.133	*0.588*	*0.379*	*0.504*	*0.847*	*0.516*
Nipple Contact: 3 Mos.	D	0.046	0.147	-0.21	0.029	-0.068	0.034
		0.783	*0.365*	*0.206*	*0.86*	*0.686*	*0.837*
Nipple Contact: 4 Mos.	D	-0.003	-0.193	-0.077	-0.016	0.143	-0.017
		0.986	*0.233*	*0.646*	*0.921*	*0.393*	*0.918*
Infant Autonomy							
Contact: 2 Mos.	F	-0.236	-0.118	-0.046	0.146	-0.077	0.114
		0.154	*0.47*	*0.783*	*0.368*	*0.645*	*0.483*
Contact: 3 Mos.	F	-0.194	-0.228	-0.092	-0.049	-0.105	-0.065
		0.244	*0.157*	*0.582*	*0.766*	*0.529*	*0.689*
Contact: 4 Mos.	F	-0.071	-0.19	-0.148	-0.176	-0.145	-0.178
		0.673	*0.241*	*0.376*	*0.277*	*0.384*	*0.272*
Explore: 2 Mos.	D	-0.135	0.055	0.128	0.156	0.061	0.112
		0.418	*0.736*	*0.443*	*0.337*	*0.717*	*0.49*
Explore: 3 Mos.	D	-0.071	-0.187	0	-0.037	-0.083	-0.071
		0.673	*0.247*	*0.999*	*0.821*	*0.621*	*0.664*
Explore: 4 Mos.	D	0.249	0.003	0.087	-0.085	-0.056	-0.113
		0.132	*0.986*	*0.603*	*0.602*	*0.737*	*0.489*
Locomotion: 2 Mos.	D	0.034	-0.154	0.294	0.154	0.179	0.217
		0.837	*0.342*	*0.073*	*0.342*	*0.281*	*0.178*
Locomotion: 3 Mos.	D	0.126	-0.189	0.301	0.041	0.183	0.065
		0.451	*0.243*	*0.066*	*0.803*	*0.271*	*0.691*
Locomotion: 4 Mos.	D	0.192	0.05	-0.03	-0.022	-0.146	0.04
		0.248	*0.757*	*0.858*	*0.893*	*0.381*	*0.808*
Infant Emotionality							
Vocalizations: 2 Mos.	F	-.628**	0.023	0.024	0.095	-0.036	0.075
		0	*0.89*	*0.888*	*0.56*	*0.83*	*0.645*
Vocalizations: 3 Mos.	F	-.591**	0.057	-0.09	-0.067	-0.174	-0.095
		0	*0.725*	*0.593*	*0.683*	*0.296*	*0.559*
Vocalizations: 4 Mos.	F	-.416**	0.067	-0.032	-0.069	-0.126	-0.083
		0.009	*0.683*	*0.85*	*0.672*	*0.449*	*0.611*

Spearman-rank correlations (*p* value provided underneath in italics). Abbreviations as follows: Faith's Phylogenetic Diversity: FPD; Duration: D, Frequency: F. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Supplemental Table 2B

		Prevotella		*Lacto.*		*Bifido.*		*Strept.*		*Faecal.*		*Caten.*		*Flexispira*	
	D/F	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo	2 Mo	4 Mo
Infant Dietary Behaviors															
Eating: 2 Mos.	D	.328*	.323*	.318*	0.176	-0.29	-0.217	-0.21	-0.112	.393*	0.004	0.283	-.403*	-0.13	0.258
		0.041	*0.045*	*0.048*	*0.277*	*0.07*	*0.178*	*0.208*	*0.492*	*0.012*	*0.981*	*0.161*	*0.041*	*0.525*	*0.202*
Eating: 3 Mos.	D	0.174	0.129	.392*	0.262	-0.12	-0.173	0.153	0.011	0.171	-0.109	0.081	-0.117	-0.18	-0.118
		0.29	*0.428*	*0.014*	*0.103*	*0.483*	*0.285*	*0.351*	*0.948*	*0.297*	*0.502*	*0.696*	*0.568*	*0.377*	*0.564*
Eating: 4 Mos.	D	0.233	-0.012	.506**	.438**	-0.19	0.028	0.227	0.076	.492**	-0.078	-0.14	-0.379	-0.07	-0.184
		0.153	*0.94*	*0.001*	*0.005*	*0.229*	*0.865*	*0.164*	*0.641*	*0.001*	*0.633*	*0.511*	*0.056*	*0.747*	*0.368*
Ventral Contact: 2 Mos.	D	0.119	-0.047	-0.15	-0.204	0.263	.398*	0.204	0.013	-0.018	0.196	0.065	0.18	0.052	0.059
		0.469	*0.773*	*0.361*	*0.207*	*0.105*	*0.011*	*0.213*	*0.939*	*0.913*	*0.226*	*0.752*	*0.38*	*0.802*	*0.775*
Ventral Contact: 3 Mos.	D	0.043	-0.264	-.372*	-0.25	0.121	.313*	-0.17	-0.001	0.013	0.081	-0.01	0.089	0.028	0.089
		0.797	*0.099*	*0.02*	*0.119*	*0.462*	*0.049*	*0.302*	*0.995*	*0.94*	*0.62*	*0.986*	*0.666*	*0.893*	*0.667*
Ventral Contact: 4 Mos.	D	0.249	0.053	-.44**	-.370*	-0.11	0.019	-0.3	-0.124	-0.077	0.084	-0.02	0.223	0.377	.466*
		0.126	*0.744*	*0.005*	*0.019*	*0.503*	*0.909*	*0.066*	*0.444*	*0.64*	*0.606*	*0.938*	*0.274*	*0.057*	*0.017*
Nipple Contact: 2 Mos.	D	0.114	-0.104	-0.016	-0.146	.325*	.434**	.329*	0.034	0.084	0.072	0.125	0.289	-0.19	-0.07
		0.491	*0.521*	*0.923*	*0.368*	*0.043*	*0.005*	*0.041*	*0.834*	*0.613*	*0.657*	*0.542*	*0.152*	*0.36*	*0.733*
Nipple Contact: 3 Mos.	D	0.139	-0.242	-0.297	-0.204	0.193	.367*	-0.19	0.021	0.001	0.08	0.096	0.107	-0.14	0.078
		0.4	*0.133*	*0.067*	*0.208*	*0.239*	*0.02*	*0.257*	*0.899*	*0.993*	*0.623*	*0.64*	*0.603*	*0.505*	*0.705*
Nipple Contact: 4 Mos.	D	.417**	0.205	-0.295	-.323*	-0.04	0.027	-0.22	-0.096	0.074	0.179	0.202	0.252	-0.09	0.35
		0.008	*0.205*	*0.069*	*0.042*	*0.798*	*0.867*	*0.18*	*0.557*	*0.654*	*0.27*	*0.322*	*0.214*	*0.674*	*0.08*
Infant Autonomy															
Contact: 2 Mos.	F	0.019	-0.039	-0.223	-0.044	-0.22	-0.275	-0.28	-0.011	0.091	-0.074	-0.04	0.086	-0.02	-0.039
		0.91	*0.812*	*0.173*	*0.786*	*0.177*	*0.086*	*0.086*	*0.948*	*0.582*	*0.649*	*0.845*	*0.677*	*0.934*	*0.849*
Contact: 3 Mos.	F	0.062	0.089	-.330*	-0.109	-0.05	-0.15	-0.28	-0.07	-0.046	0.057	-0.07	0.115	0.258	0.063
		0.709	*0.584*	*0.038*	*0.503*	*0.777*	*0.355*	*0.089*	*0.668*	*0.779*	*0.726*	*0.737*	*0.577*	*0.204*	*0.76*
Contact: 4 Mos.	F	-0.097	-0.101	-.364*	-0.149	0.238	0.08	-0.20	-0.006	-0.118	0.047	-0.12	0.157	0.201	-0.003
		0.558	*0.535*	*0.022*	*0.359*	*0.145*	*0.625*	*0.215*	*0.971*	*0.474*	*0.772*	*0.574*	*0.444*	*0.324*	*0.988*
Explore: 2 Mos.	D	.378*	0.186	0.075	0.05	-0.17	-.48**	-0.26	-0.03	0.051	-0.033	-0.24	-0.114	0.281	-0.006
		0.018	*0.25*	*0.722*	*0.76*	*0.291*	*0.002*	*0.105*	*0.855*	*0.756*	*0.838*	*0.244*	*0.579*	*0.164*	*0.976*
Explore: 3 Mos.	D	-0.083	0.15	0.067	0.033	-0.04	-0.263	0.067	0.067	0.013	-0.058	-0.03	0.084	0.107	-0.053
		0.615	*0.355*	*0.686*	*0.841*	*0.808*	*0.101*	*0.686*	*0.681*	*0.937*	*0.723*	*0.871*	*0.683*	*0.602*	*0.798*
Explore: 4 Mos.	D	-0.07	0.025	0.306	0.285	0.43*	-0.102	0.201	0.054	0.086	-0.071	0.04	-0.11	-0.20	-0.337
		0.67	*0.879*	*0.058*	*0.076*	*0.039*	*0.532*	*0.219*	*0.739*	*0.601*	*0.663*	*0.845*	*0.593*	*0.334*	*0.093*

Infant Autonomy Cont.															
Locomotion: 2 Mos.	D	-0.051	0.121	0.363*	0.297	-0.24	-.43**	-0.12	0	0.074	-0.151	-0.02	-0.133	-0.27	-0.049
		0.759	*0.456*	*0.02*	*0.063*	*0.137*	*0.006*	*0.476*	*0.999*	*0.654*	*0.353*	*0.942*	*0.516*	*0.176*	*0.811*
Locomotion: 3 Mos.	D	-0.018	.359*	.364*	0.272	-0.07	-.39*	0.192	0.003	0.021	-0.064	0.048	-0.089	-0.09	-0.02
		0.914	*0.023*	*0.023*	*0.09*	*0.665*	*0.013*	*0.242*	*0.987*	*0.899*	*0.697*	*0.817*	*0.664*	*0.648*	*0.921*
Locomotion: 4 Mos.	D	-.368*	-0.024	0.111	0.344*	0.27	-0.09	0.131	0.135	0.085	-0.002	0.099	-0.039	-.45*	-0.355
		0.021	*0.885*	*0.5*	*0.03*	*0.097*	*0.579*	*0.427*	*0.405*	*0.608*	*0.988*	*0.629*	*0.851*	*0.022*	*0.075*
Infant Emotionality															
Vocalizations: 2 Mos.	F	-0.228	-0.035	-0.115	0.235	-0.31	-0.15	-0.07	0.256	-0.077	-0.055	-0.16	0.379	0.321	-0.193
		0.162	*0.831*	*0.487*	*0.145*	*0.056*	*0.354*	*0.688*	*0.111*	*0.641*	*0.736*	*0.433*	*0.056*	*0.11*	*0.345*
Vocalizations: 3 Mos.	F	-0.304	-0.042	-0.143	0.072	-0.28	-0.046	-0.13	0.242	-0.205	0.054	-0.14	0.017	0.203	-0.335
		0.059	*0.796*	*0.387*	*0.658*	*0.086*	*0.777*	*0.435*	*0.133*	*0.21*	*0.742*	*0.508*	*0.935*	*0.32*	*0.095*
Vocalizations: 4 Mos.	F	-0.248	-0.05	-0.223	0.066	-0.17	-0.083	-0.09	0.252	-0.125	0.132	-0.06	-0.213	-0.29	-.45*
		0.128	*0.761*	*0.172*	*0.686*	*0.289*	*0.611*	*0.586*	*0.116*	*0.449*	*0.417*	*0.766*	*0.296*	*0.145*	*0.02*

Supplemental Table 2B. Genus abundance and mother-infant observations. Spearman-rank Correlations (p value provided underneath in italics). Abbreviations as follows: Lactobacillus: Lacto., Streptococcus: Strep., Bifidobacterium: Bifido., Faecalibacterium: Faecal., Catenibacterium: Caten., Duration: D, Frequency: F. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Supplemental Text 1: Description of the Human Intruder Paradigm & Normative Responses

The HIP consists of 3 two-minute baseline periods, interspersed with a 2-minute No Eye Contact condition, and a 2-minute Stare condition. A baseline period separates the profile and stare conditions to reduce habituation to the intruder. During the first baseline, monkeys are alone in the room and are given time to acclimate to the presence of the video camera. Although there is a great deal of individual variation in behavioral response on this test, infants tend to respond to the initial separation with an increase in exploration and locomotion [56,57]. In the No Eye Contact condition, a human intruder enters the room and looks toward the wall, presenting the monkey with their facial profile while avoiding eye contact with the monkey. This condition is meant to emulate a potential social threat in which the threat does not yet notice the subject. Monkeys typically attempt to remain inconspicuous by freezing and reducing vocalizations and locomotion in this condition. In the Stare condition, the intruder orients to make direct eye contact with the monkey. Eye contact is a signal of dominance and threat in many primate species, including rhesus. and this condition typically elicits aggressive or submissive behaviors from the juvenile such as barking, lip smacking, and freezing.

Supplemental Text 2: Congruence of HIP with IBAS & Mother-Infant Dyad Observations

There was, in general, concordance across the HIP and components of emotional reactivity measured through the IBAS and mother-infant observations, and deviations from the expected pattern of behavior in the HIP corresponded to differences in emotionality and behavior earlier in life. Although there was substantial individual variation in behavioral response, infants tend to react to the initial separation from their peer group with an increase in activity during the baseline, specifically in exploration and locomotion [56,57]. Among our animals, levels of activity appear to be trait-like and are consistent over time. Inactivity during the baseline condition of the HIP at 12 months of age was predictive of elevated inactivity and more frequent attempts to reestablishment maternal contact during the 3 and 4-month mother-infant observations (3mos.: r=.44, p=.009; r=.31, p=.069; 4mos.: r=.39, p=.021; r=.46, p=.005). It has been hypothesized that diminished activity during the baseline condition of the HIP may be effectively similar to behavioral inhibition and freezing behaviors, and suggest a more anxious temperament [56]. Congruently, the infants who locomoted less and remained in the back of the cage during the HIP baseline condition, and in the absence of any social threat, were found to be generally more emotionally reactive at the 2-week IBAS. On average, they startled more strongly in response to an unexpected auditory stimulus (r=.42, p=.016), vocalized more frequently when separated from their mother (r=.36, p=.029) and their predominant state was more often fearful and agitated (r=.32, p=.068). These behavioral profiles were sustained during the social threat; less activity during the baseline condition predicted less locomoting and more inactivity during the stare condition. Together, these

findings indicate that inactivity has the potential to mask a more emotionally reactive temperament, which under conditions of stress may manifest as behavioral inhibition.

Following the baseline period, monkeys are exposed to the intruder's profile meant to emulate a potential social threat in which the threat does not yet notice the subject (non-threat condition). Monkeys typically attempt to remain inconspicuous by reducing both locomotion and vocalizations and retreating to the back of the cage during this condition. In contrast to the baseline, infants that were more active during the non-threat condition displayed more emotionally reactive traits. Specifically, infants that engaged in more locomotion and exploration of the novel environment in response to the intruder's profile were more vocal (r=.42, p=.01), froze more frequently (r=.34, p=.038), spent more time in the front of their cages (r=.40, p=.016), and oriented themselves toward the intruder (r=.45, p=.006), all of which suggest a greater preoccupancy with the potential occurrence of a social threat. This reactive response to the presence of the intruder was maintained in the stare condition. The propensity toward a more anxious temperament is trait-like, manifesting as emotional reactivity as early as the 2-week behavioral assessment. Infants engaging in excessive vocalizations of fear during both the break and non-threat conditions had exaggerated responses to tactile stimulation during the IBAS (r=.39, p=.026; r=.36, p=.042). Being more active and exploratory during the profile conditions also corresponded with more contact with the mother at 2 and 3 months (r=.31, p=.062; r=.40, p=.015). This suggests that these infants matured toward autonomy more slowly and were less confident in departing from their mother [54].

While not exemplative of outward behavioral reactivity, Kalin et al. have suggested that when excessive, the duration of freezing and of orienting to the intruder reflect behavioral

inhibition and hypervigilance, which can be characteristic of an emotionally reactive phenotype [107]. This hypothesis is supported by studies reporting that individual differences in threat-induced freezing stable over time and are positively correlated with the metabolic activity of the amygdala and bed nucleus of the stria terminalis and are predictive of higher basal cortisol levels [41,56]. Among our monkeys, freezing behavior was common in both intruder conditions and corresponded to a more exaggerated startle response at 2 weeks of age (Profile: r=.40, p=.021; Stare: r=.33, p=.06). Likewise, excessive responses to tactile stimulation during the IBAS were predictive of longer durations of vigilance to the intruder during the stare condition (r=.45, p=.009). In sum, the results indicate that higher ratings of reactive emotionality at 2 weeks of age and less independence during the first several months of life may be predictive of a more emotionally reactive response to a social threat at 12 months of age.

Finally, in the Stare condition, the intruder makes direct eye contact with the monkey; this condition typically elicits aggressive or submissive social behaviors from the infant such as barking, lip smacking, and freezing. Animals who engaged with the intruder using less fearful behaviors generally showed less emotionally reactive behavior during the IBAS. For instance, infants who demonstrated aggression through frequent barking during the stare condition were more passive during the IBAS and tended to respond to unexpected stimuli with lower intensity (r=.46, p=.007; r=-.32, p=.075, respectively). Infants engaging in frequent bouts of lip-smacking, a signal of submission or appeasement, also exhibited more emotional regulation at 2 weeks of age and were easier to console (r=.40, p=.020) and soothe while being separated from their mother (r=.38, p=.027). This secure attachment was apparent throughout the nursing period; frequent lip-smacking at 12 months of age corresponded to more frequent behaviors indicative

of autonomy and less contact with the mother during the 4-month observations (r=.35, p=.039). Lip-smacking was also negatively correlated with the frequency of freezing during both the intruder profile and stare conditions, suggesting the social behavior is characteristic of a more passive and less inhibited temperament. Interestingly, lip-smacking frequency also corresponded to the infants' ability to focus and attend to visual and auditory cues at 2 weeks of age. Infants engaging in more submissive vocalizations were more attentive (r=.47, p=.006) and demonstrated more orienting toward visual stimuli for longer durations (r=.40, p=.022; r=.35, p=.045, respectively). Significantly, calm attentiveness in infancy is an indicator of more mature development of emotional regulation [36]. There was no significant relationship between aggressive and fearful vocalizations with early-life orienting abilities suggesting that considering the intention of infant vocalizations may provide further cues when evaluating their relationship with temperamental traits in neonates.

Supplemental Text 3: Construction of Hip Temperament Phenotypes

After confirming that the HIP captured distinctive individual differences in reactions to a social threat, three composite variables were created to profile aspects of emotionality and overall temperament. Two behavioral indices of emotional reactivity were employed: behavioral inhibition and behavioral reactivity. Behavioral inhibition was constructed from the summed durations of orienting toward the intruder and freezing during the profile condition. Behavioral reactivity was calculated by summing the frequency of screams vocalized and fear grimaces, and threats shown during the stare condition. Because animals who are behaviorally inhibited have been described as showing less activity in the absence of threatening stimuli [58], a third factor comprising environmental exploration and locomotion during the baseline condition was created. The sum of locomotion, exploration, and cage position during the profile condition was additionally considered to elucidate inappropriate responses to the potential threat. However, the variance accounted for by this composite score overlapped with the Activity factor and, therefore, was not pursued further (See Supplemental Table 3 for factor correlates). Infants were ranked with respect to each composite variable and those ranking in the top quartile of the behavioral inhibition and emotional reactivity composite factors and in the bottom quartile of the activity factors were rated as behaviorally inhibited, behaviorally reactive, and inactive, respectively. A review of the prior literature on the HIP indicates that these three extracted factors are largely reflective of the constructs others have suggested to underlie behavioral responses to a social threat, namely outward emotionality, activity, and behavioral inhibition [38,56,58].

Supplemental Table 3: Human Intruder Composite Variable Correlations

	F1 Motor Activity	F2 Emotional Reactivity	F3 Behavioral Inhibition	F4 Inappropriate Response to Non-Threat
F1 Motor Activity	1			
F2 Emotional Reactivity	-.018	1		
F3 Behavioral Inhibition	.182	.096	1	
F4 Inappropriate Response to Non-Threat	.418*	-0.113	0.094	1

The *r* coefficients were determined with the use of Pearson correlation analysis. The table shows the relative independence of the first 3 factors (i.e., by the lack of significant associations). F4 Inappropriate Response to Non-Threat was dropped due to non-independence. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Chapter 4: Maternal and breast milk influences on the infant gut microbiome, enteric health and growth outcomes of Rhesus monkeys (*Macaca mulatta*)

Originally published as:

Rendina DN, Lubach GR, Phillips GJ, Lyte M, Coe CL. Maternal and breast milk influences on the infant gut microbiome, enteric health and growth outcomes of Rhesus Monkeys. *J Pediatr Gastroenterol Nutr*. 2019, published online May 9;
DOI: 10.1097/MPG.0000000000002394

ABSTRACT

Objectives: Gut bacteria play an essential role during infancy and are strongly influenced by the mode of birth and feeding. A primate model was used to investigate the benefits of exposure to the mother or conversely the negative impact of early nursery rearing on microbial colonization.

Method: Rectal swabs were obtained from rhesus macaques born vaginally, breastfed, and mother-reared (MR, N=35) or delivered primarily via cesarean-section, formula-fed, and human-reared (HR, N=19). Microbiome composition was determined by rRNA gene amplicon sequencing at 2, 4 and 8 weeks of age and KEGG orthologs used to assess influences on functional metabolic pathways in the gut. Growth trajectories and incidence of diarrheic symptoms were evaluated.

Results: The microbial community structure was different between MR and HR infants with respect to phylogeny and abundance at all 3 ages. When examining dominant phyla, HR infants had a higher *Firmicutes-to-Bacteroidetes* ratio. At the genus level, breast milk-dependent commensal taxa and adult-typical genera were more abundant in MR infants. This difference resulted in a corresponding shift in the predicted metabolic effects, specifically for microbial genes associated with metabolism and immune function. HR infants had faster growth trajectories ($p<0.001$), but more diarrheic symptoms by 6 months postnatal ($p=0.008$).

Conclusions: MR infants acquired adult-typical microbiota more quickly, and had higher levels of several beneficial commensal taxa. Cesarean-delivered and formula-fed infants had different developmental trajectories of bacterial colonization. Establishment of the gut microbiome was

associated with an infant's growth trajectory, and implicated in the subsequent vulnerability to *Campylobacter* infections associated with diarrhea in infant monkeys.

What Is New:

- The microbiome of human-reared infant monkeys is characterized by a higher ratio of *Firmicutes*-to-*Bacteroidetes*, a profile associated with metabolic disorders in humans.
- The stimulatory effect of breast milk on commensal propagation was more evident for *Bifidobacteria* than *Lactobacilli*.
- The gut microbiome during early infancy is predictive of future risk for *Campylobacter jejuni* infections in rhesus monkeys.

INTRODUCTION

Birth is an abrupt and transformative event, transitioning the neonate from relatively sterile, protected uterine conditions into the external world. The full significance of the rapid microbial colonization that begins during delivery and continues postpartum through exposure to microorganisms from the mother and rearing environment is just beginning to be appreciated. These microbiota have a critical role in sustaining the infant's health by promoting intestinal homeostasis, stimulating and educating the immune system, providing protection against pathogens, influencing host metabolism, and may even contribute to the emergence of different behavioral phenotypes [1,2]. Though the foundations of the microbiome can be influenced by prenatal conditions, the microbial community structure is largely impacted by delivery mode and the infant's diet, especially parental decisions about breast-feeding or bottle feeding with formula [3–5]. Delivery by Caesarean section and formula feeding disrupt the vertical transmission of microbes from mother to infant [5–7], and both factors have also be associated with the development of long-term health consequences, such as metabolic disorders and atopic conditions and asthma [8–11]. The underlying mechanisms are not well understood, but there is growing evidence linking microbial inoculation during vaginal delivery and exposure to breastmilk with sequential microbial patterns of gut colonization in later infancy [7].

In addition to the obvious nutritional differences, breast milk contains numerous proteins and prebiotic oligosaccharide substrates, and provides viable bacteria that compete with pathogens for adherence to the intestinal mucosa and epithelial surfaces [12,13]. Among infants in Western countries, and also in the infants of closely-related nonhuman primates,

breast-feeding favors the propagation of commensal taxa, including *Bifidobacterium*, whereas formula-fed infants have an overrepresentation of *Clostridium* [14,15]. Predominance of these commensal microbes is predictive of lower overall microbial diversity within the infant gut, and may offer protection against infection with enteric pathogens [16,17]. In the primate species evaluated in our studies, the primary pathogens of concern are *Campylobacter jejeuni* and *Shigella flexneri*, both of which are known to be more common in infant monkeys that were raised by humans in a nursery-setting with formula [18]. While there have been prior microbiology studies of mother- and human-reared monkeys, our analyses used current culture-independent 16S sequencing methods to more definitively characterize the gut microbiome across the first 2 months of life. We were particularly interested in the initial acquisition of microbes by 2 weeks of age and then the ecological succession during the period of active nursing while microbes would be vertically transmitted from the mother.

Prior research in infant monkeys has also indicated that there is an association between the infant's gut microbiome and host metabolism [19]. Thus, we conducted KEGG prediction analyses to investigate how the bacterial taxa present in the infant's gut might influence metabolic activity. Infants' weight gain trajectories were additionally monitored in order to evaluate how early-life diet might influence the rate of growth. Previous analyses of human-reared infant monkeys in nursery settings have typically found that they initially grow more rapidly than do the infant monkeys breastfed by their mothers [20]. It continues to be important to more fully understand the ramifications of delivery mode and parental decisions about infant feeding because, despite recommendations from the World Health Organization, cesarean delivery has become increasingly more common in both developed and developing

countries and only 40% of American infants are exclusively breastfed until 6 months of age [21,22]

The current study employed a nonhuman primate model to investigate the benefits of exposure to the mother and breast milk for microbial colonization. We hypothesized that bacterial colonization would be distinctive between rearing conditions with mother-reared infants having a higher abundance of several beneficial commensal taxa, while the human-reared infants might evince a higher ratio of Firmicutes-to-Bacteroidetes, a profile associated with metabolic disorders in humans [23,24]. Finally, because microbial colonization can affect digestive efficiency and host metabolism, we expected that there would be differences in growth rates, and potentially in the incidence of diarrheic episodes across the first year of age.

METHODS

Subjects. Fifty-four infant rhesus macaques (*Macaca mulatta*) were generated from healthy, multiparous mothers at the Harlow Center for Biological Psychology and Wisconsin Primate Research Center. Thirty-five were housed with their mothers and exclusively breast-fed (MR). All were full-term vaginal deliveries. Nineteen were fed formula and reared by humans (HR) for one month in isolate incubators mimicking a Neonatal Intensive Care Unit (NICU) setting, with 13 delivered by cesarean-section. Infants from both rearing conditions were randomly selected to be representative of the population born between 2016-2018.

No infants were treated with antibiotics during the sampling period; however, the mothers of 5 MR infants had been administered antibiotics perinatally. Infants were first weighed at a mean 3.65 (± 0.61) days, and then regularly at 2-8 week intervals. Growth was

indexed by weight, as well as growth rate between birth and 8 months. Diarrheic symptoms and treatments were recorded. In the majority of cases, to determine if *Campylobacter* was the cause, plates with selective media were inoculated (Campy CVA Medium and Charcoal Selective Medium), and streaked for isolation. Identification was made using a MALDI-TOF analyzer at the UW Veterinary Clinical Pathology Lab.

Infant rearing procedures. HR infants were fed Similac Sensitive® with OptiGro, which contains prebiotics. Formula was prepared fresh by animal care staff and was mixed according to the manufacturer's instructions. Following a soak in warm water with disinfectant, used bottles and nipples were cleaned using under hot water with a bottlebrush and were sanitized daily. HR infants started with bottle-feeding 60mL of formula 6 times daily for 2 weeks, progressing to ad libitum self-feeding from a mounted bottle. HR infants were introduced to chow soaked in formula and small pieces of fruit at 2 weeks of age. At one month, HR infants were paired with another HR infant and transferred to a stainless-steel cage. At day 30, infants were now given a bottle 5 times daily and were progressively weaned from formula by 2 months. Mothers and older infants were fed a commercial biscuit diet, supplemented with fruit. Typically, MR infants may first sample biscuits between 2-4 weeks after birth. HR infants were introduced to chow at 2 weeks of age and were progressively weaned from formula by 2 months. All protocols were approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal guidelines.

Specimen Collection, Isolation of Bacterial DNA, and Sequence Analysis. Rectal swabs were obtained between 0930-1130 at 2, 4 or 8 weeks of age. A small quantity of fecal matter was sampled using the BBL™ CultureSwab™ Collection & Transport System swab/tube (Becton Dickinson) and stored at <-60 ºC. Extraction of genomic DNA was performed using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). Purified genomic extracts were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and stored at -20 °C in 10 mM Tris buffer until sequenced. PCR amplification of the V4 variable region of the 16S rRNA gene using variable region-specific primers (515F-806R) and amplicon sequencing were performed on an Illumina MiSeq by the Argonne National Laboratory (Lamont, IL). Each sample generated an average 30,597 sequences. Sequences were demultiplexed and quality filtered through QIIME default settings, and closed reference operational taxonomic unit (OTU) picking was conducted using the GreenGenes 13_8 reference database (19) to classify and group unique sequences into OTUs based on 97% nucleotide sequence identity. OTUs with fewer than 15 reads, and two samples with fewer than 1,000 filtered sequences, were excluded from further analysis. Infant growth and health were closely monitored.

Statistical Analysis. Significance was tested by implementations in Quantitative Insights into Microbial Ecology (QIIME) [25], PAST (PAleontological STatistics) [26], and R Statistics, including ANOVA, Analysis of Similarity (ANOSIM), nonparametric t-tests (Kruskal-Wallis), and adjustments for False Discovery Rates (FDR). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (18) was utilized to correct for variable 16S copy number. Taxonomic analyses were restricted to more abundant taxa present at a

minimum 1% composition in total observations across samples and are presented at phylum and genus levels. To examine diversity indices, Faith's Phylogenetic Diversity (PD), Chao1 Index, and Observed Species were generated to examine richness (alpha diversity), and weighted UniFrac dissimilarity matrices examined for differences in microbial community structure (beta diversity) while accounting for both abundance and phylogenetic relatedness. Repeated measures ANOVA examined developmental changes in microbial composition, followed by pairwise comparisons adjusted for multiplicity. Using GreenGenes 13_5 (19) assigned OTUs, PICRUSt was employed to predict microbial metagenomes from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs in order to evaluate whether shifts in community structure influenced the functional potential of bacterial communities. Finally, linear discriminant analysis (LDA) effect size algorithm from LEfSe [28] identified microbial taxa that distinguished the two rearing conditions.

Results are presented in two ways: first comparing the MR and HR infants at the 3 age points. Serial changes in community structure for the MR infants are then described.

RESULTS

Analysis 1: Community structure. 102 rectal swabs were collected from 35 MR infants and 19 HR infants at 2, 4 and 8 weeks of age (See Supplemental Material, Table 1, for more details). The microbial diversity and composition for the 5 MR infants born to mothers administered perinatal antibiotics did not differ significantly from MR infants without antibiotic exposure. Rearing condition had a significant effect on the gut microbial community structure. Principle Coordinates Analysis for phylogenetic and abundance data revealed a distinctive clustering

based on rearing evident at each age point (Figure 1A), and permutational multivariate analysis of variance (PERMANOVA) indicated divergent microbiota at 2, 4 and 8 weeks (p=0.001, p=0.001, p=0.002, respectively). Unpaired t-test comparisons of the mean dissimilarity distances for the microbial profiles indicated that sample-to-sample phylogenetic distances were larger in HR infants by 2 weeks of age, (t=2.99; p<0.001), but the dissimilarity in community structure then shifted and was greater in MR infants at 4 and 8 weeks (t=3.29; p<0.01, t=3.60; p<0.005; Figure 1B). Overall, divergence of the microbial community structure increased in MR infants across the first 2 months of life, while decreasing among HR infants (p<0.001 and p=0.011, respectively). Averaged OTU counts for HR infants were higher than for MR infants (444.62 versus 404.89). However, there was not a large difference in the within-group pairwise phylogenetic distances (PD index; Figure 1C), sample diversity (Chao1), or total species richness (Observed OTUs) at any age point age (See Supplemental Material, Table 2, for alpha diversity metric results).

Taxonomic Abundance. Differences were evident at all taxonomic levels, but most prominent at the phylum and genus levels (Figure 2A,B). Similar to humans, the most abundant microbes were members of the gram-positive *Firmicutes* and gram-negative *Bacteroidetes* phyla. However, the *Firmicutes*-to-*Bacteroidetes* ratio was impacted by rearing, with *Firmicutes* being relatively more abundant in HR infants at 2, 4 and 8 weeks of age (p<0.001, p=0.05, p<0.001, respectively; Figure 2C). Within *Bacteroidetes*, *Prevotella* was consistently the most abundant genus in MR infants, comprising an average 9.9% compared to only 5% in HR infants at 2 weeks

of age (p=0.03). The highest *Prevotella* levels were seen in MR infants at 8 weeks (13%), an abundance similar to adult monkeys.

Conversely, the genus *Blautia* (within *Firmicutes*) was more enriched in HR infants at 4 and 8 weeks of age (10.5% vs. 3.9%, p=0.07; 9.4% vs. 4.9%, p=0.02, respectively). HR infants also had a lower relative abundance of the phyla *Actinobacteria* (Figure 2B), reflecting a significantly lower abundance of the genus *Bifidobacterium* at 2, 4, and 8 weeks of age (p<0.001, p=0.007, p=0.002, respectively). Moreover, *Bifidobacteria* diminished over time in HR infants; constituting only .2% and .15% at 4 and 8 weeks as compared to 1% at 2 weeks (p=0.018). *Lactobacilli* were also lower in HR than MR infants, but this difference was only significant at 8 weeks (p=0.017) and was small (2% vs. 3.5% abundance). At 2 weeks, the proportion of *Clostridium* was twice as high in HR compared to MR infants (1.7% vs. .8%), but the abundance decreased with age (p=0.056). The carriage rate, or percentage of infants in which the typically pathogenic *Staphylococcus* was detected was also higher among HR as compared to MR infants at 2 weeks (75% and 47%, $\chi 2$=3.48, p=0.063), but differences in prevalence diminished by 4 weeks of age (p=0.34).

Predicted shifts in functional pathways. PICRUSt predictions indicated the different community structures would be accompanied by significant shifts in several metabolic pathways, including environmental and genetic information processing, cellular processes, and metabolism (Supplemental Materials, Table 3). At Level 2 of the KEGG pathway analysis, predictions for HR infants indicated pathway increases related to energy, carbohydrate and lipid metabolism and xenobiotic biodegradation and metabolism. Conversely, MR infants evinced increases in genes

mapping to glycan biosynthesis and pathways associated with metabolism of vitamins, terpenoids and polyketides, and amino acids. KEGG predictions also projected that genes related to environmental adaption and immunity would be downregulated in HR infants, while biochemical pathways implicated in human infectious disease would be enhanced.

Infant Growth and Health Trajectories. Growth curve analysis was used to analyze weight gain through 8 months of age. The overall growth trajectory of HR infants was faster [$F(1,78.77)=14.52$, $p<0.001$; Figure 3]. Despite having similar birth weights ($p=0.22$), HR infants weighed an average 188 g more at 2 months [$F(1, 51)=38.76$, $p<0.001$] and 88 g more at 4 months [$F(1, 51)=7.60$, $p<0.01$]. Weight differences diminished by 6 months after infants had transitioned to solid food. The phylogenetic richness of the microbial community structure (PD index) at 8 weeks tended to be positively associated with infant weight at the time of sampling ($r=.350$, $p=0.08$), and was significantly correlated with subsequent weights at 4, 6, and 8 months of age ($r=.433$, $p=0.027$; $r=.408$, $p=0.043$; and $r=.418$, $p=0.034$, respectively). More microbial richness at 8 weeks was also associated with a faster growth trajectory ($r=.404$, $p=0.041$). When comparing gut health between rearing conditions, more incubator-reared and formula-fed infants were treated for both acute and chronic diarrhea by 6 months of age (7 of 19 HR vs. 3 of 35 MR, $\chi2=6.53$, $p=0.011$; See Figure 4). Differences in vulnerability to enteric pathogens, identified as *C. jejuni* in 70% of cultured stool, continued to persist after infants were weaned to solids (6 of 19 HR vs. 2 of 35 MR, $\chi2=4.69$, $p=0.03$).

Analysis 2: Serial evaluation of MR infants. Seventeen MR infants were sampled at all 3 age points permitting a serial analysis of developmental change within each infant. Linear mixed effect modeling revealed that community richness was characterized by an increase in diversity that stabilized at 8 weeks ($F(2,16)=3.175$, $p=0.055$). However, there was no difference in beta diversity over time. Despite the relative stability of these diversity indices, the relative abundance of genera did change. *Prevotella*, the most common genera, increased to 22.8% ($F(2,32)=3.29$; $p=0.05$), with higher levels at 8 weeks than at 2 and 4 weeks ($p=0.053$; $p=0.011$, respectively). *Bifidobacteria* were second most abundant, comprising an average 15.5% of identified taxa, and the levels were stable during active nursing. *Lactobacillus* abundance diverged, increasing to 6.7% by 4 weeks of age, but decreased by nearly half as other taxa became prominent at 8 weeks ($F(2,32)=5.82$, $p=0.007$). KEGG orthologs analyses did not identify any age-related predictions in microbial gene function that survived correction for FDR in this smaller subset of MR infants.

DISCUSSION

Our findings concur with prior research demonstrating that bacterial colonization is strongly affected by early rearing conditions. Vaginally-born infants were colonized by microbial populations more closely related to the maternal reproductive tract and gut, and dominated by *Prevotella* and *Lactobacillus* [5,7]. This natural colonization was perturbed if delivered by cesarean-section and instead the infants show a delayed colonization by *Bacteroides* and *Bifidobacterium* and may have been enriched in skin microbiota, including *Staphylococcus*, thus resembling the gut microbial composition of human infants exposed to a NICU [6,29,30].

Breastfeeding is a second major factor influencing the establishment of the microbiome during the neonatal period. It favored the growth of commensal *Bifidobacteria* and *Lactobacillus*, whereas the formula-fed infant monkeys had an overrepresentation of *Clostridium* [15,31,32]. However, while *Bifidobacteria* species can comprise nearly 81% of the gut microbiota in vaginally-born and breastfed human infants [4], levels were considerably lower in the monkey [19]. This species difference may be due to the higher concentrations of *Bifidobacterium*-promoting oligosaccharides present in human milk. The ratio of oligosaccharides to lactose is 1:1.26 in humans, whereas it is only 1:6 in the rhesus monkey [33,34]. We expected a higher abundance of *Lactobacilli* in MR infants due to vertical transmission and stimulation from prebiotic constituents in breast milk. However, other studies have found that this effect is not always clearly evident [5,35]. In addition, the absence of a larger difference when compared to *Lactobacilli* abundance in the HR infant may reflect the recent addition of prebiotic glycans to the particular formula they consumed. Fructooligosaccharides, including those present in Similac Sensitive® formula, can significantly increase both *Lactobacillus* and *Bifidobacterium*. However, *Lactobacilli* growth is less dependent on oligosaccharides [36] and a dose-dependent stimulating effect on growth has been seen only for *Bifidobacteria* [37]. The effect of early feeding regimens on *Lactobacilli* may also be delayed. A prior study on nursery-reared rhesus infants fed formula with prebiotics indicated they ultimately had substantially lower levels of *Lactobacillus*, but not until after they were weaned onto solid foods [31].

Despite significant differences in overall composition and individual taxa, we did not see large differences in phylogenetic richness between the two rearing conditions. However,

phylogenetic diversity was predictive of weight gain in both conditions as the infants began to consume solid foods. Other studies have found diminished diversity in HR infant monkeys at weaning and during the transition to solids [31], so it is possible that a diminished community richness only becomes apparent later. Our HR infants did, however, evince a higher ratio of *Firmicutes*-to-*Bacteroidetes*, due in part to an abundance of *Blautia*, a profile associated with more efficient absorption of calories and metabolic disorders in humans [23,24,38]. This difference resulted in a corresponding shift in predicted metabolic pathways based on functions attributed to these microbes, including increased genes associated with carbohydrate and lipid metabolism. KEGG predictions were substantiated by larger and faster weight gains in HR infants. A differential effect of early rearing on growth rate has been reported previously for nursery-reared monkeys as well as in formula-fed human infants [19,39]. Though this difference was diminished after weaning, rapid weight gain during infancy has been correlated with later risk for adult obesity, dyslipidemia, and insulin resistance in humans [40].

In addition to influencing host metabolism, gut bacteria can sustain infant health by providing protection against pathogens [1,2]. When clinical records were reviewed at both 6 and 12 months, the HR monkeys exhibited more diarrheic symptoms with verified *C. jejuni* infections during and subsequent to the transition to solid foods, confirming the benefits of breastfeeding for providing sustained protection against enteric pathogens [41]. *C. jejuni* is associated with gut dysbiosis and the abundance of *Campylobacter* is predictive of the overall microbial composition, even in asymptomatic infant and juvenile monkeys [42–44] . Further demonstrating the importance of maternal influences, a prior study documented that a high-fat maternal diet prior to birth had a protracted effect on the abundance of *Campylobacter* present

without symptoms in infant monkeys [45]. Previous studies have also documented differences in the immune responses of MR and HR monkeys [19,46], which are likely associated with the protective qualities of lactic acid bacteria and *Bifidobacterium*, commensal strains that were more abundant in MR infants and known to enhance intestinal epithelial barrier function [47,48]. Oligosaccharides in breast milk can selectively stimulate the propagation of these microbes, as seen in the predicted upregulation of genes involved in the metabolism of host glycans in the MR infants [36,49]. Through these mechanisms, breast milk can be protective against diarrheal disease. Specifically, it has been shown that probiotics can discourage the growth and intestinal adhesion of *C. jejuni, Clostridium,* and other pathogenic organisms [50]. Moreover, *Prevotella*, the predominant genera in MR monkeys, may also have a protective role because it contributes to the production of fermentation enzymes responsible for short-chain fatty acids (SCFA) [51]. These enzymes and SCFA are critically important for the regulation of immune responses. Collectively, these findings reaffirm the view that there is a critical early window for initiating the trajectory to gut health.

While our findings concur with many prior studies, limitations should be acknowledged. The HR condition differed in more than one way from the MR condition, because it involved both formula-feeding and early rearing in an incubator. However, both factors can co-occur in human infants. Infants born premature or delivered through cesarean-section are more likely to be admitted to the NICU and their mothers are less likely to breastfeed, or to delay breastfeeding initiation [52,53]. Many of the HR infants were being reared in this manner because the biological mother had a cesarean delivery. There were repeated attempts to reunite the HR infant with its mother during the first week of life, a practice that could be

considered to mimic the bacterial exposure that might occur during the 'skin-to-skin contact' encouraged for preterm NICU infants [54]. Future research will have to more selectively vary the dietary and social variables that differed between the two rearing conditions. Ours was designed to discern more maximal microbial differences that might occur in the absence of the mother and breast milk. For this type of investigation, a monkey is preferable to rodent models because breast milk composition and gut maturity are different in species with altricial neonates [33,34]. Though ours is one of the larger studies to examine factors influencing the infant microbiome in monkeys, the sample size did limit statistical power. It precluded stratified analyses to identify interactions between delivery mode and feeding method. We were also underpowered to conduct a serial analysis of the changing microbiome in individual HR infants, but did have a sufficient number of MR infants for repeated measures analysis (see Supplemental Materials, Table 1). Finally, the gut microbiome is not the only outcome known to differ between MR and HR monkeys; the mother also stimulates neural and behavioral development [55].

In summary, breastfed infant rhesus exposed continuously to maternal sources of bacteria more quickly acquired microbiota typical of adults and had higher levels of several beneficial symbionts. The findings concur with the view that there is a biological expectancy that a mother will be present to provide a sustained microbial inoculation. Many clinical, personal and economic factors contribute to obstetrical decisions about delivery mode and parental decisions about feeding regimens after birth, but the influence on the infant's gut microbiome should be taken into consideration. While the improved composition of formula, including prebiotic factors, now allow it to more closely resemble mother's milk, we still need

to advance our understanding of its prebiotic functions. The initial community structure of the infant's microbiome can have long-term metabolic and physiologic effects influencing the developmental trajectory to adult health in animals and humans.

Acknowledgments

Acknowledgements are due to the staff of the WNPRC who provided specimens from the HR infants (P51OD011106) and to Alexandra Proctor for help with DNA extractions and advice on the 16S sequence analysis. All bacterial DNA was extracted and processed by the laboratory of GP and ML at ISU.

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Chapter 4: FIGURES & TABLES

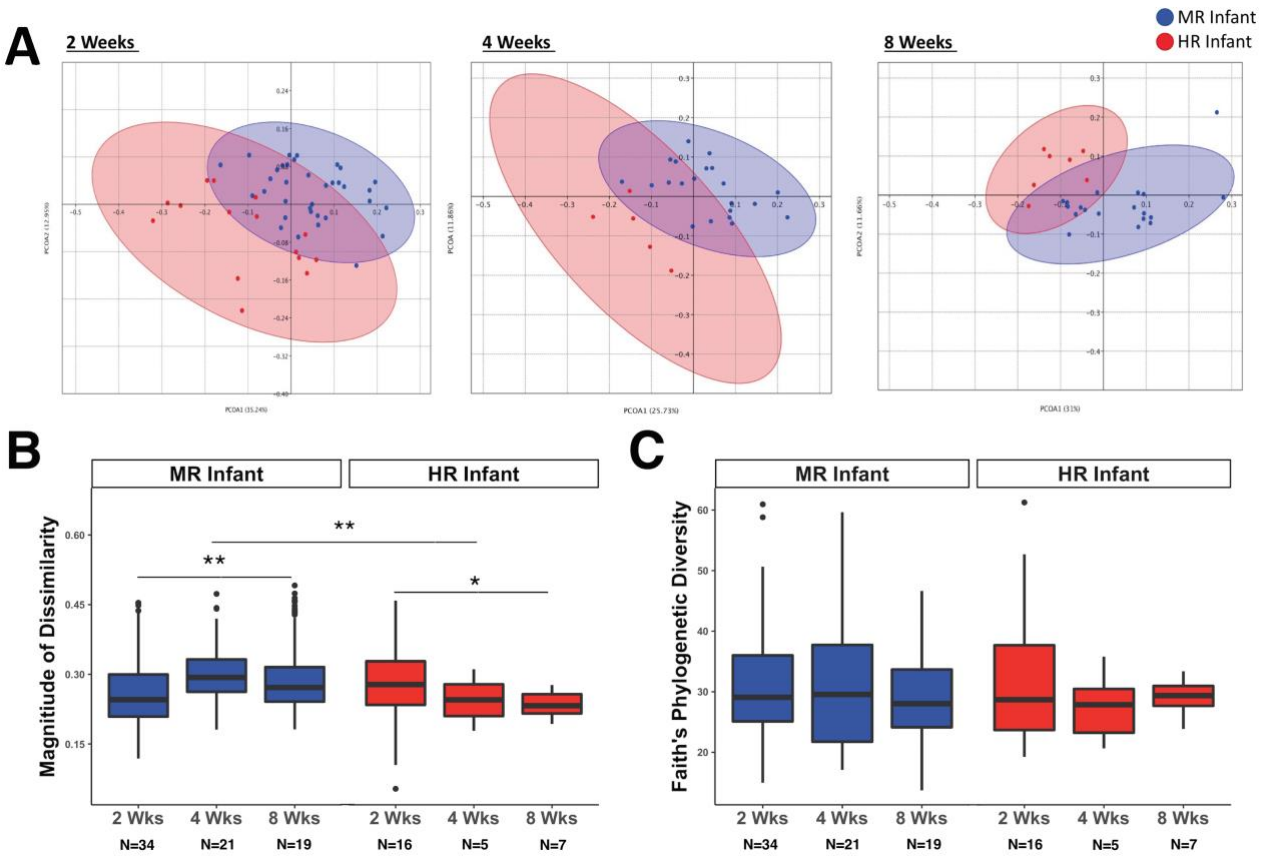


Figure 1 Microbiome Diversity Indices across the first two months of life. **A)** Analyses of beta diversity based on weighted UniFrac distances between samples. Principal coordinates analysis with ellipses representing 95% confidence intervals shows clear separation of microbial profiles between MR and HR infants at 2, 4, and 8 weeks. **B)** Inter-individual distances illustrate the magnitude of dissimilarity in the profiles, which was significantly larger in HR infants at 2 weeks of age, but was lower than the microbial diversity across all MR infants at 4 and 8 weeks of age. **C)** Further categorization based on the phylogenetic analogue of taxon richness revealed no effect of rearing condition at any age point. Values are: box, median; whiskers, 25 and 75% quartiles; lines, 1.5 times the interquartile range. Outliers are illustrated by circles. *, p<.01; **, p<.005.

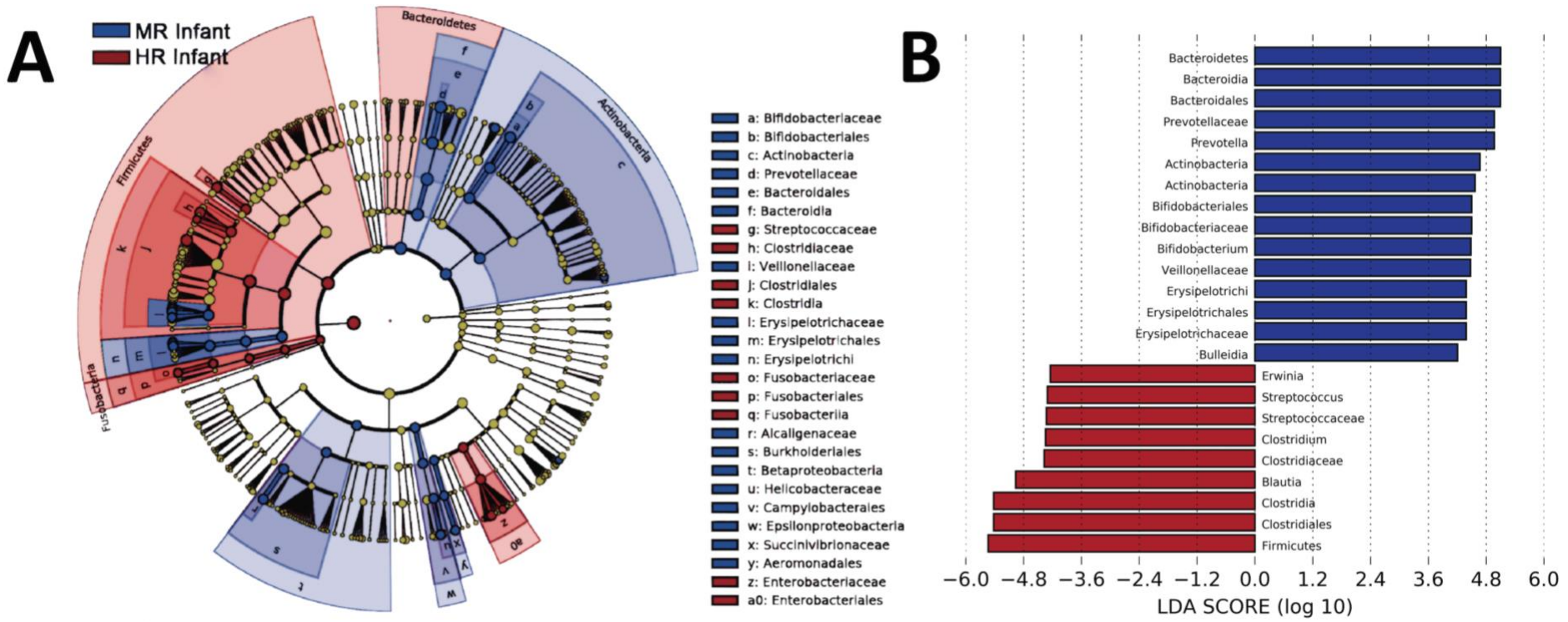


Figure 2 Rearing environment predicted differences in taxonomic composition of gut microbiota. **A)** Taxonomic cladogram plotted from LEfSe analysis of 16S sequences from all time points. (Blue) MR infant enriched taxa; (Red) HR infant enriched taxa. Brightness of each dot is proportional to its effect size. **B)** Histogram of Linear Discriminant Analysis scores computed for features that distinguish between MR and HR infants which estimates the effect size of each differentially abundant feature (all LDA scores >4.0).

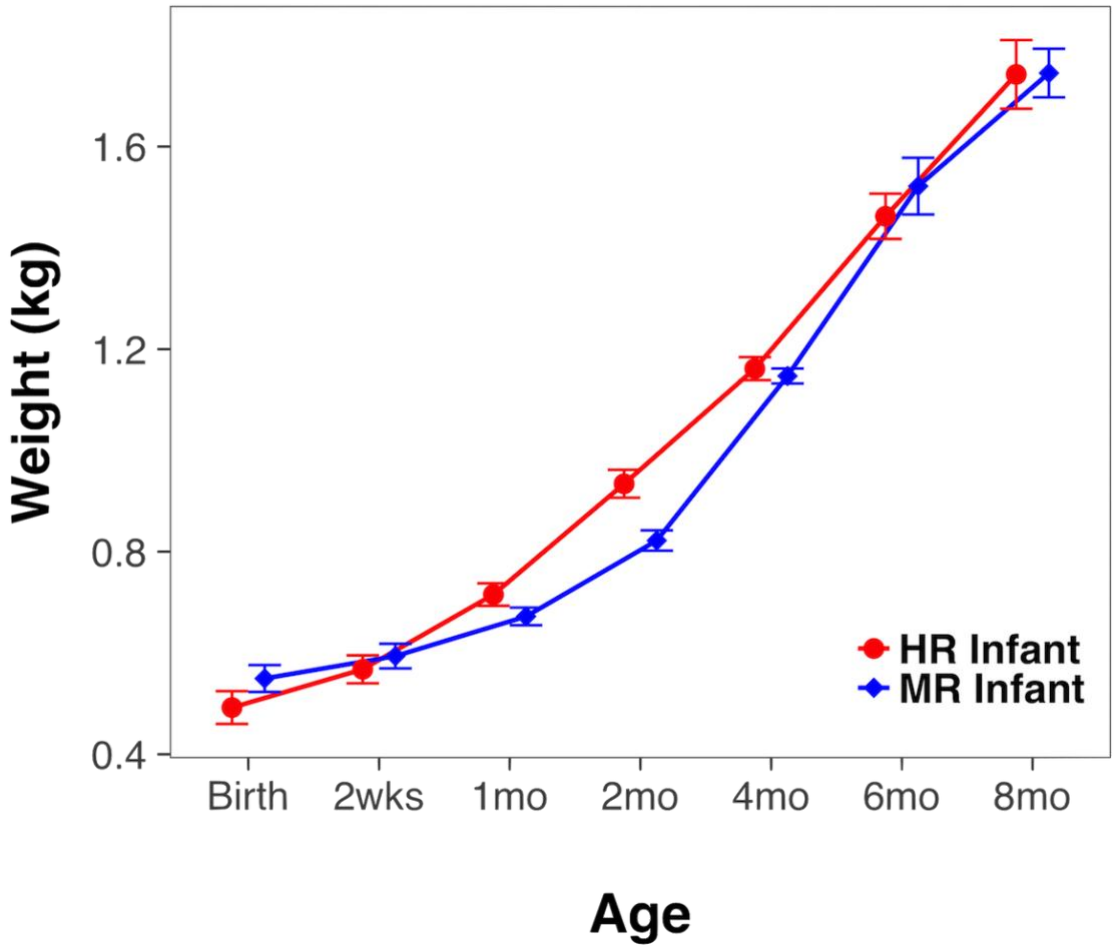


Figure 3 Infant growth differed by rearing condition. In keeping with formula-feeding and a unique gut microbial structure, HR infants grew significantly faster until 6 months of age, despite similar birth weights in both rearing conditions. All infants were consuming solid food by 4 months of age when growth rates converged.

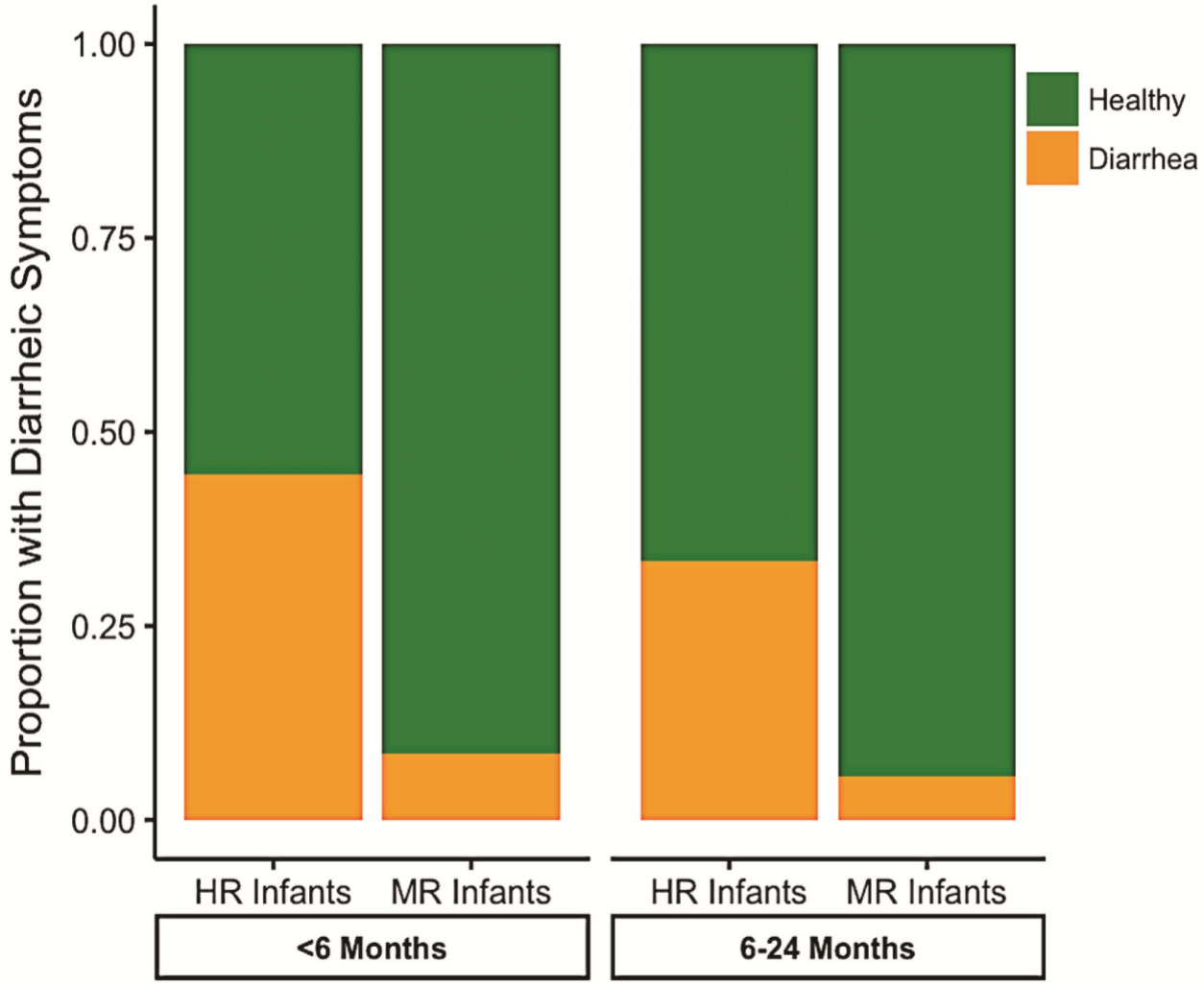


Figure 4 Prevalence of diarrheic symptoms. HR infants were more likely to be treated for both acute and chronic diarrhea by 6 months of age and the increased susceptibility to enteric pathogens continued during the 6-month period following weaning. Clinical stool cultures were run on 73% of infants exhibiting symptoms; *Campylobacter jejuni* was most common pathogen identified.

CHAPTER 4: SUPPLEMENTAL MATERIALS

Table, Supplemental Content 1. Infant Recruitment Descriptives

	Total		Female Infants		Male Infants	
Age	MR	HR	MR	HR	MR	HR
2 weeks	34	16	20	8	14	8
4 weeks	21	5	12	2	9	3
8 weeks	19	7	12	3	7	4

Table, Supplemental Content 2. Alpha Diversity Metrics

	2 Weeks			4 Weeks			8 Weeks		
Alpha Diversity Metrics	*p*	MR Mean	HR Mean	*p*	MR Mean	HR Mean	*p*	MR Mean	HR Mean
Faith's Phylogenetic Diversity	0.83	31.50	32.27	0.58	30.63	27.60	0.85	28.52	29.12
Chao1 Index	0.57	489.11	531.41	0.72	457.97	494.25	0.17	437.79	528.32
Observed OTUs	0.56	420.12	453.19	0.72	403.71	431.80	0.26	390.84	448.86

Supplemental Table 3. Significant differences predicted for metabolic pathways associated with the microbiota of Mother and Human-Reared infants*

KEGG Prediction	Test Statistic	*p*	FDR-*P*	MR Mean	HR Mean	Difference
Environmental Information Processing						
Signal transduction	20.56	0.000	0.000	213,446.61	278,115.08	64,668.47
Membrane transport	11.39	0.001	0.003	1,858,851.12	2,091,898.12	233,047.00
Cellular Processes						
Cell motility	17.61	0.000	0.001	264,134.76	357,417.04	93,282.28
Cellular processes & signaling	16.81	0.000	0.001	631,089.28	699,712.36	68,623.08
Cell growth and death	5.99	0.014	0.019	86,701.01	78,069.84	8,631.17
Organismal Systems						
Excretory system	16.41	0.000	0.001	1,625.78	2,517.24	891.46
Environmental adaptation	12.44	0.000	0.002	23,713.73	26,061.76	2,348.03
Immune system	11.01	0.001	0.003	14,732.84	13,770.64	962.20
Nervous system	7.32	0.007	0.011	16,079.82	15,362.52	717.30
Circulatory system	6.50	0.011	0.015	1,110.34	1,263.84	153.50
Human Diseases						
Infectious diseases	14.82	0.000	0.001	62,751.11	65,904.16	3,153.05
Neurodegenerative diseases	5.16	0.023	0.029	19,478.16	19,588.24	110.08
Metabolic diseases	5.10	0.024	0.029	18,132.22	15,709.72	2,422.50
Metabolism						
Lipid metabolism	13.43	0.000	0.002	425,769.61	456,215.64	30,446.03
Energy metabolism	11.72	0.001	0.003	948,442.86	958,682.16	10,239.30
Metabolism of cofactors & vitamins	10.38	0.001	0.003	710,506.43	689,044.68	21,461.75
Carbohydrate metabolism	9.92	0.002	0.004	1,673,406.01	1,740,094.60	66,688.59
Terpenoids & polyketides	9.61	0.002	0.005	273,118.54	264,845.20	8,273.34
Metabolism of other amino acids	9.37	0.002	0.005	251,763.42	242,611.20	9,152.22
Glycan biosynthesis & metabolism	9.22	0.002	0.005	426,098.57	366,852.00	59,246.57
Amino acid metabolism	8.78	0.003	0.006	1,542,558.74	1,521,755.72	20,803.02
Xenobiotics biodegradation & metabolism	8.64	0.003	0.006	248,805.08	256,821.84	8,016.76
Nucleotide metabolism	6.56	0.010	0.015	698,936.45	630,544.24	68,392.21
Secondary metabolite biosynthesis	6.03	0.014	0.019	153,533.15	141,316.84	12,216.31
Genetic Information Processing						
Transcription	11.33	0.001	0.003	424,546.05	467,090.48	42,544.43
Folding, sorting & degradation	10.53	0.001	0.003	405,204.04	389,156.56	16,047.48
Replication and repair	6.94	0.008	0.013	1,514,143.88	1,380,323.64	133,820.24
Translation	5.84	0.016	0.020	980,135.35	884,161.76	95,973.59

***KEGG predictions are representative of all time point**

CONCLUSIONS

Identification of a developmental succession in the infant microbiome

The colonization of the gut microbiome follows a predictable developmental trajectory in the infant rhesus monkey. Data presented within this thesis indicate that, consistent with reports in human infants albeit at a more rapid rate, the gut of the infant monkey undergoes dynamic change, and acquires more evenness and complexity in the community structure with age [1]. Following birth, the gut of the infant monkey is colonized by facultative anaerobes, which have been reported to shift the intestinal environment to the anaerobic conditions preferred by obligate anaerobes [2]. As the infant diet later transitions from breastmilk to solid foods, the gut microbiome acquires greater abundances of Firmicutes and Bacteroidetes, while the abundances of Actinobacteria and Proteobacteria are diminished. This shift in microbial structure occurs around 6 months of age when infants are weaned naturally from their mother and are relocated to housing in small social groups with peers through husbandry practices. Co-housing with other weanlings facilitates the sharing of microorganisms, thus contributing to the decrease of inter-animal variation and the convergence in microbial profiles that was observed by 1 year of infant age [3]. Although the 12-month period of sample collection did not capture the complete maturation process, the phylogenetic richness and diversity of the infant microbiomes, and their predicted metabolic functionality, were comparable to that of their mothers by 8 months of age after they had been weaned and transitioned to a diet of exclusively solid foods. In some infants, perturbations in the microbial succession were found to

be associated with subsequent reductions in microbial richness and a delayed development of an adult-like microbiome.

In contrast to their infants, rhesus macaque mothers exhibited stability in the microbial composition over the duration of the 6-month lactation period, which has several implications. First, this temporal stability in the adult females suggests that additional factors beyond exposure to the maternal gut microbiome are responsible for the microbial succession of the infant gut. Additionally, stability maintained by the adult gut highlights the importance of early colonization events on the microbiota that will be sustained in later life. Due to priority effects in community assembly, earlier colonizers, such as those acquired from parents and the environment, have the potential to influence the subsequent successional patterns of the microbiota community [4]. The capacity for transient occupiers of the infant gut to impact sequential microbial colonization suggests that, despite finding little evidence of direct correspondence in the gut communities of mother-infant pairs, there is likely an effect of the infant's early life experiences on microbiota developmental trajectories.

The significance of diet and the transition from suckling to solid foods

Infant nutrition appears to have a major impact on the microbiota found in the immature gut, with the cessation of breastfeeding and relocation away from the mother into peer-housing being associated with the most dramatic changes seen in the composition of the gut microbial community. During the early nursing period, the gut microbiota of infants was characterized by a higher rate of microbial diversification and a predominance of

Bifidobacterium and taxa within *Bacteroidetes*, which metabolize milk oligosaccharides with high efficiency [5,6]. After weaning from the mother and the adoption of a diet consisting exclusively of solid foods, the microbial community structure within the infant gut stabilized and acquired a more adult-like composition, which was marked by increases in *Lactobacillus* and *Streptococcus*. These associations were further reinforced in the observational study where it was found that suckling behaviors indicative of nursing and consumption of breastmilk were associated with a higher abundance of *Bifidobacterium* within the infant gut, whereas shifts in gut microbial richness and diversity were apparent as infants were observed to become more independent and adopt a more varied diet. Specifically, the abundances of *Lactobacillus* and *Faecalibacterium* appear to be closely associated with the increased consumption of commercial monkey biscuits, which increase dietary fiber intake, and the supplemental enrichment foods provided periodically as part of the routine husbandry.

Furthermore, our findings suggest that differences in early life experiences and consummatory behavior have a synergistic effect on the microbial succession and maturation within the gut. In the experiment described Chapter 4, disrupting exposure to maternal sources of microbes and to breast milk through the manipulation of rearing conditions delayed the acquisition of an adult-typical microbiota and resulted in lower levels of several commensal symbionts. The prebiotic potential of breastmilk was particularly apparent in the finding that rhesus infants delivered by caesarean section and then formula-fed were host to lower abundances of *Bifidobacterium, Prevotella* and *Bacteroides.* These atypical early rearing conditions additionally resulted in an overrepresentation of the pro-inflammatory taxa *Clostridium*, the proliferation of which is usually suppressed by milk oligosaccharides [7].

Prominence of Prevotella & Lactobacillus as microbial signatures of the mature rhesus gut microbiota

Like humans, the rhesus monkey is colonized primarily by microbes within the Bacteroidetes and Firmicutes phyla. However, while members of the genus *Bacteroides* and taxa within the order of *Clostridiales* have been reported to be a major and functionally significant component of the gut microbiota of humans consuming Western diets [8], the gut microbial community of adult female macaques was dominated by *Prevotella* and *Lactobacillus*. With the exception of the neonate at the time of birth, *Prevotella* was also the most prevalent taxa within the infant gut and abundances increased over the first 6 months of age and nursing. Members of the Bacteroidetes phylum are generally determined by environmental and dietary exposure [9,10], and the preponderance of *Prevotella* within the rhesus gut has been suggested to be linked to their consumption of a diet rich in fiber, reflecting the potential of this taxa for facilitating carbohydrate catabolism and plant glycan degradation [11]. *Prevotella* abundance within the infant gut shows a clear correspondence to maternal levels only when assessed shortly after birth. Notably, in addition to its predominance within the gut, *Prevotella* has been reported to be the most common genus within the reproductive tract of female nonhuman primates after *Lactobacillus* [12,13], which suggests that the *Prevotella* detected in the infants could have been acquired in part during the process of being born by vaginal delivery. Further support for this hypothesis comes from the finding that infants born through caesarean section and formula-fed had lower abundances of *Prevotella* during the first 8 weeks of life.

In contrast to *Prevotella*, *Lactobacillus* levels were relatively stable during periods of exclusive breastfeeding and increases were seen later in conjunction with dietary transitions to solid foods. These patterns of microbial succession are dramatically different from those reported in the gut of human infants, where *Lactobacillus* displays a striking increase in breastfed infants over the first 6 weeks of life and has the potential to reach around 70% of the microbial composition [14]. This discrepancy between primate species may have several origins. While *Lactobacillus* is the dominate genus within the conserved primate vaginal microbiota, the vaginal microbial composition of non-human primates is highly diverse, and *Lactobacilli* abundance is more sparse in comparison to levels seen in human females [15,16], suggesting that the lower colonization seen in rhesus infants could be the result of less vertical transmission through vaginal delivery. Additionally, other research has shown that the *Lactobacillus* spp. present in rhesus infants were also different from those found in human infants and their mothers' milk, and are less efficient consumers of the oligosaccharides found in breastmilk [17]. This species difference is further reflected in the finding that differences in the abundance of *Lactobacillus* between infants that were vaginally delivered and breastfed and those that were born via caesarean section and fed formula were minor and only significantly different at 8 weeks of age. By 8 weeks of age, most infants are beginning to sample solid foods and no longer exclusively consuming breastmilk; *Lactobacillus* within the rhesus infant gut appears to be particularly responsive to the consumption of solid food. Taken together, these results suggest that while vaginal seeding and breast milk constitute major sources of *Lactobacilli* for the human infant, rhesus infants may be colonized with *Lactobacilli* in a different manner.

Microbiota succession associated with infant physical and neurodevelopmental outcomes

Compositional shifts in the infant gut microbiota are also indicative of changes in the predicted metagenomic activity of the microbiota within the gut and may have important implications for host metabolism and developmental health. Within this dissertation, it was observed that infants progressing to a more diverse and even microbial composition, and hence a more mature profile, had faster growth trajectories, both in weight gain and skeletal growth, and larger overall brain volumes at 12 months of age. Gut microbes contribute significantly to host nutrition and energy resources and appear to be a key factor contributing to infant growth. Specifically, a higher abundance of Firmicutes during the period of peak nursing was a microbial signature of more efficient growth, reflecting host metabolism [18]. Conversely, when this vertical transmission of bacteria from mother to infant was not as evident, especially during the period of breastfeeding, it was associated with atypical growth trajectories and subsequent vulnerability to infection with *Campylobacter jejuni* later in infancy. Although our manipulation of early life rearing conditions did not allow for a definitive determination of causal relationships, others have reported that reduced exposure to maternal sources of microbes and breastfeeding during early development leads to anomalous gut microbiota profiles, reductions in immune competence, and later in adulthood, an increased incidence of metabolic disease [19–21].

Because others have reported that the gut microbiota during infancy are associated with the emergence of behavioral phenotypes [22–24], research within this dissertation also investigated the association between microbial community structures and infant emotionality and behavior. Although testing with the Human Intruder Paradigm (HIP) did prove useful for

characterizing emotional reactivity, the primary determinants of temperament derived from the HIP were not found to be strongly related to microbial community structure or the abundance of milk-consuming symbionts, either early in life or contemporaneously when the HIP tests were conducted. These findings differ from research done in germ-free rodent models, which has demonstrated clear effects of the absence of a gut microbiota on the stress reactivity and neurodevelopment of the host [25–28]. It is possible that shifts in the relative abundance of single taxa or even the overall microbiota community structure do not have as pronounced an effect on emotionality in otherwise healthy infants. It may also be the case that it is easier to detect a simultaneous effect on the gut microbiome and emotionality when one perturbs the system and microbiota are manipulated, such as with germ-free or specific pathogen-free mice. It is well known that there are multiple regulators of gut homeostasis in the healthy individual that might prevent one taxa from changing the whole community structure or from being salient enough to affect the emotionality of the host [29]. Associations between the commensal microbes and infant behavior were more apparent when the infant was only 2 weeks of age, and when analyzing the measures acquired with the Infant Behavioral Assessment Scale (IBAS). Similarly, more associations were evident with the naturalistic observations of the mother-infant dyad throughout the nursing period. Specifically, behaviors associated with poor emotion regulation were more common in infants with a more diverse microbiota at 2 weeks of age, characterized by lower abundances of *Bifidobacteria*. Additionally, at an older age during the nursing period, less autonomy was observed in infants exhibiting lower abundances of *Lactobacillus*.

Rhesus infant as a model of microbial ecology in the primate gut and future directions

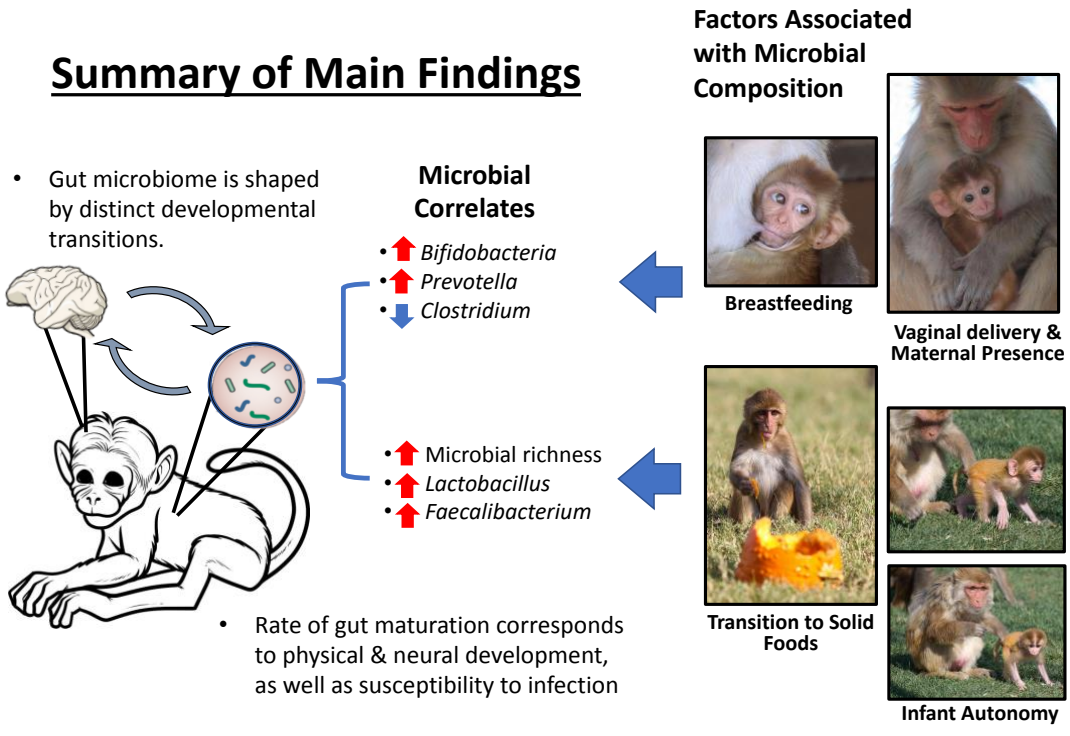
The work presented in this dissertation provides further evidence that a combination of infant diet, behavior, and early environmental experiences are critical factors that shape the microbiota present in the infant gut. These findings have translational relevance for the rearing of human infants and demonstrate the value of the rhesus monkey as an informative model for research on the successive changes in gut microbiota in early life. The rhesus monkey may be particularly useful as a model for infants from developing countries due to the enriched abundance of *Prevotella* within their microbiota and their susceptibility to several pathogens associated with diarrheal diseases, such as *Campylobacter jejuni* and *Shigella flexneri*, which disproportionally affect children in countries with poorer hygiene and contaminated water supplies [30]. Rhesus monkeys have long been used in biomedical research due to genetic, physiological, and neurodevelopmental similarities to humans [31–33], and the standardized husbandry practices used in the care of monkeys in laboratory settings allow for more control of critical variables (such as diet, hygiene, and size of the social network) than is possible in research with humans. However, the studies within this dissertation did identify several substantive differences between non-human primates and humans that future research should take into consideration. In order to optimally use the rhesus infant as a model of human infant gut development, the origins and consequences of these differences between primate species need to be better understood.

Specifically, infant monkeys exhibit a more diverse gut from a younger age than do human infants, and there are important species differences in the relative abundancies of

several commensal taxa. Because a diverse gut microbial community is more resistant to the invasion of opportunistic pathogens [34], rhesus infants may exhibit less suseptibility to environmental conditions that might foster dysbiosis and their gut community may be more difficult to manipulate. One potential experimental option might be to shift the microbial composition of the rhesus infant to more closely resemble that of the human infant through oral administration of commensal microorganisms or via controlled dietary regimens. For example, it has been demonstrated that within the reproductive tract of adult female rhesus it is possible to promote a sustained increase in their *Lactobacillus* profile through either provision of a prebiotic suppository that is conducive to *Lactobacilli* growth or via probiotic administration of *Lactobacilli* to better model the healthy vaginal milieu of women [35,36]. However, before remodeling the gut of the rhesus infant, further understanding of the fitness effects of the specific microbes reported as commensal and beneficial in humans is needed. The gut microbiota is often described as having co-evolved with the host [37], and while many taxa can perform similar metabolic functions, and activate the same metabolic pathways, different microorganisms can have different needs for acidity, oxygen and growth conditions. Therefore, the needs and functionality of these taxa needs to be taken into consideration before attempting to manipulate the overall community structure.

Future studies are also needed to better characterize the temporal changes in milk composition and metabolic activity as lactation progresses and the young monkey develops. Epidemiological evidence has demonstrated that breastfeeding has many beneficial functions in promoting infant health, and the provision of protective immunity, including being the primary source of secretory IgA that coats the surface of the oral cavity and gut [38]. In humans, the

protective benefits of breastfeeding are also explained by its influence on the establishment of the infant gut microbiota, specifically targeting and facilitating the growth of several commensal species, including both *Bifidobacteria* and *Lactobacilli* [14,39,40]. However, there has still been relatively little research on how the non-nutrient constituents of breastmilk modulate the gut microbiota composition of the infant, and even less on how it affects gut and overall host health in the rhesus monkey. In order to better understand the origins of some of the species differences in gut colonization that have been described in this thesis, these non-nutrient components of breast milk and their relationship with the microbial succession of the gut of the rhesus monkey are currently being investigated through collaborative work with Dr. Concepcion Remoroza at the National Institute of Standards and Technology. By identifying the prebiotic potential of rhesus breast milk and its capacity to stimulate gut microbiota maturation, these findings have the potential to further advance the rhesus as a translational model for the study of maternal influences on the infant microbiota and gut development.



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