EARLY EXPOSURES, BODY COMPOSITION AND THE GUT MICROBIOME

FROM BIRTH TO THREE YEARS: THE RELATIONSHIP OF EARLY-LIFE EXPOSURES, BODY COMPOSITION AND THE GUT MICROBIOME

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TITLE: From Birth to Three Years: The Relationship of Early-Life Exposures, Body Composition and the Gut Microbiome

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Lay Abstract

The gut microbiome, consisting of trillions of microbes, is crucial for childhood growth and immune health. The microbiome undergoes rapid changes during infancy and reaches maturity by three years. Our study monitored 245 mother-infant pairs from birth to three years to investigate the association between the infant gut microbiome and factors such as delivery place, maternal parity, intrapartum antibiotic usage, growth, and body composition.

We show that delivery place, parity and antibiotic usage are significantly associated with gut microbiome diversity. Infants born to mothers with higher parity and who are not exposed to antibiotics during labour show elevated levels of beneficial bacteria, such as *Bifidobacterium*, in the first three years. A relationship between growth trajectory and the early infant gut microbiome could not be established in this cohort. These insights into underexplored early-life factors can guide strategies to promote a healthy gut microbiome from an early age.

Abstract

The gut microbiome is essential for immune system development and infant metabolic health. The first three years of life are critical for colonization and maturation of the infant gut microbiome. Our study involves 245 full-term, low-risk mother-infant pairs recruited by midwifery practices at McMaster University. It spanned from birth to three years, with data collected from birth records, study questionnaires, anthropometric measures, and stool samples processed via 16S rRNA sequencing. We investigated associations between the infant gut microbiome and [1] early-life exposures such as delivery place, maternal parity, and intrapartum antibiotic prophylaxis and [2] growth (BMIz) and body composition (FMI and LMI). We characterized the following aspects of the infant gut microbiome longitudinally over the first three years of life: (1) alpha diversity, within sample diversity, (2) beta diversity, among sample diversity, and (3) bacterial taxonomy.

Our study reveals significant associations between early-life exposures and gut microbiome diversity during infancy. Infants delivered at home show elevated levels of *Bacteroides* at ten days postpartum. Parity is the strongest predictor of beta diversity variation, with infants showing higher levels of *Bifidobacterium* in their first year. Infants exposed to intrapartum antibiotics showed reduced *Bifidobacterium* and increased *Streptococcus*, which persisted for up to three years. Growth and body composition show minimal associations with gut microbiome diversity. BMIz, FMI and LMI are inversely related to alpha diversity at five months of age, but no associations existed at other timepoints. FMI and BMIz are associated with beta diversity at 12 weeks and 5 months, respectively. While the microbiota correlates with growth and

body composition measures at five months, its predictive utility diminishes by three years, emphasizing the lasting association of early-life exposures on gut microbiome variation. Future work should focus on elucidating the underlying microbial mechanisms and developing microbiome-focused interventions aimed at improving infant health.

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List of Abbreviations

ADP	Air displacement plethysmography
ASV	Amplicon sequencing variant
BMIz	Body mass index z score
C-section	Caesarean section
DXA	Dual-energy X-ray absorptiometry
FMI	Fat mas index
GBS	Group B *Streptococcus*
GI	Gastrointestinal
HMO	Human milk oligosaccharide
IAP	Intrapartum antibiotic prophylaxis
IBD	Inflammatory bowel disease
LMI	Lean mass index
MUAC	Mid-upper arm circumference
'Multips'	Infants of multiparous mothers
NFIL3	Nuclear factor, interleukin 3 regulated
'Nullips'	Infants of nulliparous mothers
PCoA	Principal coordinate analysis
PERMANOVA	Permutational analysis of variance
RDP	Ribosomal Database Project
SCFA	Short-chain fatty acid
VIF	Variance inflation factor

Declaration of Academic Achievement

This is a declaration that Johan Carballo completed the content of the research presented in this thesis. The research study was designed by Dr. Katherine Morrison, Dr. Jennifer Stearns, and Dr. Eileen Hutton as part of the Baby and Mi research grant, and all contributed to the review and completion of this thesis. Additional contributors to the revision and editing of this thesis include Dr. Jonathan Schertzer, Dr. Michael Surette and Julia Simioni. To the best of my knowledge, the content of this thesis does not infringe on others' copyrights.

1.0 Introduction

1.1 The Infant Gut Microbiome

A diverse set of living organisms, such as bacteria, fungi, and archaea, comprise the human microbiome at various locations in the body[1]. Specifically, the gut microbiota refers to the community of these microorganisms in the gut[2]. The gut microbiome extends this concept by encompassing the collection of all genomes from these microorganisms and their functions and interactions within the gut[2]. The gastrointestinal (GI) tract is one of the body's largest and most dynamic ecosystems, with microbial colonizers playing critical roles in host health and disease acquisition[4–6]. Chronic diseases such as Type 2 diabetes mellitus, inflammatory bowel syndrome (IBD), and obesity have been linked to colonization patterns of the gut microbiota in early life[7–10]. Antibiotic use in early life is associated with less microbiota diversity and the development of allergic diseases such as eczema and asthma[11–13].

As the microbiome develops during infancy, microbe-host interactions are also established, which aid in creating a healthy immune system[14,15]. Infancy is a critical period for gut microbiome development, whereby an adult-like microbiome is observed by three years of age[14,16]. There are three phases of microbiome progression in early life: developmental (months 3-14), transitional (months 15-30), and stable ($\geq$ 31 months)[17]. Many factors contribute to the characteristics of the gut microbiome in early life. These include delivery place, mode of delivery, breastfeeding, formula feeding, solid food introduction, maternal parity, and intrapartum antibiotics[5,18]. While an 'optimal' gut microbiota has not been described, these early-life factors may impact the gut microbiota and consequentially influence long-term health outcomes[14,19].

1.2 Factors Related to the Infant Gut Microbiome

Several early-life factors may influence microbial colonization and succession. Delivery place, whether hospital or home, may influence microbial exposures encountered by the neonate during childbirth and shape the composition of the gut microbiome[5,19,20]. Hospital environments harbour distinct microbial communities compared to home environments and are characterized by higher levels of potentially pathogenic bacteria and lower levels of beneficial microbes[21,22]. Infants born in hospital settings may be exposed to different microbial reservoirs during childbirth and early infancy compared to those born at home, and this may impact microbial colonization patterns and health outcomes[21–23]. Hospital-born infants exposed to a medicalized environment show an increased presence of hospital-associated microbes, such as *Enterococcus*, potentially affecting their immune development and disease susceptibility[22,24]. Conversely, home births promote more diverse microbial colonization, including higher levels of *Bifidobacterium* and *Lactobacillus*, which could offer better health protection[23–25].

Mode of delivery, whether vaginal or Caesarean-section (C-section), is a critical factor associated with the early infant gut microbiome. Babies delivered by C-section may experience delayed immune system development, increasing their risk of developing immune system disorders such as asthma and allergies in childhood[6,20]. This heightened risk is partly due to the limited diversity of early bacterial colonizers when prophylactic antibiotics are also administered during labour[11]. This distinct microbial profile in C-section births includes an increased abundance of skin-associated and opportunistic pathogens like *Clostridium difficile*, which may predispose these infants

to a higher risk of allergies, asthma, and obesity[13,20,26,27]. In contrast, vaginally delivered babies rapidly acquire microbial communities resembling those found in the maternal birth canal, characterized by high levels of *Lactobacillus* and *Bacteroides*[14,19,28]. The early colonization of these beneficial bacteria can positively influence the infant's immune system development and overall health[14,19]. Although the initial disruption in colonization by *Bacteroides* in C-section-delivered babies may eventually stabilize, it can have lasting impacts on long-term health and development[28,29].

While delivery mode is associated with the first year of microbiome colonization, differences in microbiome composition based on delivery mode seem to dissipate as other environmental factors start impacting microbial succession[6,16]. For instance, by six months of age, microbial succession is driven by breastfeeding[30]. A characteristic increase in *Bifidobacterium* is observed for breastfed individuals[6,19]. Breastmilk contains human milk oligosaccharides (HMOs), a known immune system primer that allows a *Bifidobacterium*-rich community to thrive[19]. Formula-fed infants show lower abundances of *Bifidobacterium*, with formula food fostering the colonization of pro-inflammatory taxa[6,31]. Another critical milestone is solid food consumption, which introduces fibre in the diet and a shift to taxa more capable of carbohydrate metabolism and vitamin biosynthesis[32,33]. By three years of age, the gut microbiota of children is stable and resistant to perturbation[6]. However, an unbalanced diet during infancy may cause gut dysbiosis, whereby beneficial commensal bacteria are lost in favour of pathogenic microbes[32,33]. This may also trigger a pro-inflammatory response and immune system dysregulation[33]. A well-balanced gut microbiota plays a significant role in metabolism, nutrition, immune system regulation, growth development, and disease prevention[5,6,14].

While research has established that delivery mode is strongly associated with the infant gut microbiome, several critical questions remain[34,35]. Does elective versus emergency C-section have differential effects on microbial colonization and diversity? Is the infant's microbiota shaping solely due to the initial bacterial colonizers influenced by delivery place and mode, or does an underlying mechanism dictate microbiome succession in early life? The long-term effects of delivery place and mode on the gut microbiome remain underexplored for three years and beyond into childhood. Whether these early-life factors have prolonged effects on microbiome development or merely set the stage for initial microbial colonization is uncertain. Understanding the impact of delivery place and mode of delivery on the infant gut microbiome during the first three years of life is essential for elucidating microbial succession patterns and their implications for infant health and disease.

1.3 Parity and the Maternal Gut Microbiome

The maternal gut microbiome may play a crucial role in maternal and infant health[36,37]. Microbial metabolites produced in the maternal gut can be transmitted to the placenta and fetal tissues, aiding in the immune development of the fetus[36,38]. Infants also acquire protection from metabolic disease due to the acquisition of maternal-derived short-chain fatty acids (SCFAs) that cross over via the placenta[36,39]. Maternal parity, defined as the number of pregnancies a woman has experienced, has emerged as a possible determinant of maternal and infant gut microbiomes[36,40]. Parity can influence maternal immune responses, hormonal profiles, and breastfeeding practices, which may affect microbial transmission to the neonate during childbirth and breastfeeding[36,37]. While research on the association between the infant gut microbiome and parity is still

emerging, some linkages have been identified between parity and the maternal gut microbiome[36,37,40].

A study explored how maternal adaptations to pregnancy, influenced by factors like pre-pregnancy body mass index (BMI) and gestational weight gain, affect the maternal gut microbiome[37]. They suggest that maternal 'ecological memory' influenced by parity can modulate the impact of gestational factors on early microbial colonization in infants[37]. Parity shapes maternal gut microbiota adaptations during pregnancy[37]. It influences maternal gut microbial colonization, underscoring its significant role in early-life microbial dynamics and potential implications for infant microbiome health[37].

Remodelling of the maternal gut microbiome during pregnancy is influenced by parity, with significant differences in microbiota trajectories observed between multiparous and nulliparous mothers[36]. A swine model study examined how parity influences gut microbiome changes during pregnancy, revealing distinct trajectories for multiparous compared to nulliparous mothers[36]. Even one previous pregnancy significantly alters the gut microbiota's trajectory during subsequent pregnancies. High-parity mothers experience more abrupt microbiome shifts early in pregnancy, while low parity and nulliparous mothers show more gradual change[36]. This indicates that the maternal gut microbiota adapts differently based on prior pregnancies, potentially influencing microbial transmission to the infant during childbirth[36]. Despite the significant association of maternal parity with the gut microbiome composition of sows, the study found no direct correlation between the maternal gut microbiota and early infant gut colonization[36]. This suggests that while maternal factors significantly drive

offspring microbiome composition, the mechanisms likely differ from direct vertical transmission of specific microbial taxa[36,37].

Furthermore, maternal parity may influence mode of delivery and infant feeding practices, thus, impacting the composition and diversity of the infant gut microbiome[20,35,36]. Nulliparous women may be more likely to undergo C-section delivery or initiate breastfeeding later or for shorter durations than multiparous women, leading to differences in microbial colonization patterns in their infants[20,35,36]. These variations in the infant gut microbiome may have implications for immune development, metabolism, and long-term health outcomes[20,35,36].

While current research is focused on linking parity with the maternal gut microbiome, its role in the developing infant gut microbiome still needs to be understood[36,37]. It is unclear how the number of previous pregnancies from the mother influences microbial colonization in newborns and whether changes in maternal health and microbiome composition across successive pregnancies play a role. The potential interactions between parity and other factors, such as delivery mode and maternal diet, in shaping the infant's gut microbiome have yet to be well elucidated. Additionally, we do not yet understand how parity affects the infant's microbiome—whether it works through changes in the mother's microbiome or directly by colonizing the infant's gut. Understanding the correlation between maternal parity, maternal health, and the infant gut microbiome is crucial for identifying modifiable factors affecting microbial colonization patterns and health outcomes in early life.

1.4 Intrapartum Antibiotic Prophylaxis

Intrapartum antibiotic prophylaxis (IAP) for Group B *Streptococcus* (GBS) is a critical intervention in modern obstetrics to minimize neonatal GBS infection[41–43]. Approximately 25% of pregnant people carry GBS, which can be passed to the baby during labour and delivery[44,45]. Mothers typically show no symptoms of GBS; thus, rigorous screening tests are conducted to prevent the baby from infection, as it can be lethal in some cases. IAP is administered to mothers who test positive for GBS during routine screening, have a previous history of early-onset GBS infection or show several other risk factors such as fever during labour, preterm labour, and C-section delivery[44]. While the benefits of preventing infection are clear, administering antibiotics during labour can significantly perturb the maternal microbiome and alter the colonization of the infant gut microbiome[45,46]. Antibiotics can modify the maternal vaginal and fecal microbiota composition and diversity—key sources for seeding the infant's gut—thereby disrupting microbial transmission from mother to infant[41,45].

IAP for GBS perturbs colonization of the infant gut microbiome, resulting in increased *Enterobacteriaceae* and reduced *Bifidobacterium*[41,42,45]. Such shifts in microbial composition are consequential, potentially affecting immune development and metabolism and predisposing infants to long-term health challenges[41,47]. IAP has been documented to have lasting associations on microbial diversity and increased risks of asthma, obesity, and altered immune responses in infants exposed to antibiotics during childbirth[26,42,45]. The timing and duration can influence the extent of these alterations. The specific antibiotics used, with prolonged or repeated exposure can likely cause more significant and lasting changes in the infant's microbial gut colonization[48,49].

It is crucial to develop interventions that promote microbial resilience and support the restoration of a balanced microbiome in infants exposed to antibiotics during labour. This understanding will inform clinical practices, guiding the judicious use of antibiotics in childbirth and implementing measures to preserve neonatal microbial health, thereby supporting optimal immune development and metabolic outcomes in early life.

1.5 Infant Growth and Body Composition

Microbiome composition may influence childhood growth and development[43,50]. Several anthropometric measures, such as body mass index (BMI), height, and mid-upper arm circumference (MUAC), are commonly used to track healthy growth trajectories[51–53]. The gut microbiota may affect growth trajectories and body composition, and some of the proposed mechanisms are by enhancing satiety, promoting energy storage in adipose tissue or activating systematic inflammation[43,50,54,55].

Several animal studies have been conducted to uncover the relationship between the gut microbiota and body composition[55–57]. To understand the molecular mechanisms that link the gut microbiota and body composition, one study suggested that the microbiota regulates body composition through the circadian transcription factor NFIL3[55]. They show that the microbiota influences *Nfil3* expression by decreasing lipid deposition in mice on a high fat diet[55]. Another study found that mice exposed to protein-energy undernutrition showed an altered gut microbiome and loss of key pathways for harvesting energy from their diet[56,57]. In a follow-up study, the undernourished mice were refed, and while their growth was restabilized, their intestinal microbiota and metabolomes remained significantly perturbed[56,57].

The association of maternal parity extends beyond microbial colonization to include significant implications for offspring growth and development. In a study with vervet monkeys, researchers hypothesized that infant growth might be influenced by vertical-transmitted gut microbes[40]. Despite lower milk production, offspring from low-parity females grew larger, possibly due to reduced early-life gut microbiome diversity and dominance of *Bacteroides fragilis*[40]. This suggests that a specialized milk-oriented microbiota promotes infant growth[40]. This also highlights the possible evolutionary role of *B. fragilis* in primate development[40]. These findings illustrate how parity influences microbial colonization, milk-oriented microbiota transmission, and infant growth, revealing adaptive mechanisms crucial for early-life development in primates[40].

While no consensus has been reached on the bacterial species influencing growth and body composition in children, some studies have attempted to associate the presence and abundance of taxa in children with different anthropometric growth measures[52,53,58,59]. Children with normal BMI typically have higher *Bifidobacterium*, whereas overweight children show higher numbers of *Staphylococcus*[58]. Infants with higher BMI-z scores have elevated levels of *B. fragilis* and reduced numbers of staphylococci between 3 and 26 weeks of age[52]. The KOALA cohort study observed that infants with a high-fibre diet had a positive correlation between high BMI z scores and *B. fragilis* numbers, while infants with a low-fibre diet showed an inverse trend[59]. The influence of gut microbiota concerning malnutrition and poor growth has also been studied in human subjects. A study involving Malawi twins discordant for kwashiorkor determined that a perturbed microbiome was observed for the malnourished twin[60]. While nutritional interventions may cause temporary weight gain in underweight children, they don't immediately change the microbial community structure,

emphasizing the importance of maintaining a mature gut microbiome for long-term healthy growth[50,61].

The characteristic microbiota of overweight/ obese individuals may be associated with other confounding factors, such as diet and antibiotics, influencing microbiome composition[7,62] Exposure to antibiotics in early life has been shown to alter gut microbiota composition and increase adipose tissue and short-chain fatty acid (SCFA) levels[47,63]. While SCFAs can increase microbial energy harvest, they are also known to activate peptide hormones that stimulate satiety; thus, the exact role of SCFAs on obesity is still unclear[50,63]. A Danish study using 28, 354 mother-child pairs investigated the effect of antibiotics and the risk for weight gain[43]. They determined that antibiotic use in the first six months of life increased the risk of the infant being overweight if the mother was of average weight[43]. Conversely, the risk of being overweight decreases if the mother is obese or overweight[43]. The microbiome may uniquely regulate weight gain, as evidenced by different colonization patterns that result in altered body compositions[43,50]. Some obese individuals show a lower abundance of *Akkermansia muciniphila*, which may assist in protecting the host from inflammation and excess adipose deposition[50,64]. Correlations between microbiota and body composition have been suggested, yet very little is still known about the direct effect of the microbiome on infant trajectories of fat mass changes over time.

2.0 Project Overview

2.1 Rationale

In this study, we focus exclusively on bacterial communities, using 16S rRNA sequencing to determine alpha diversity, beta diversity and taxonomy of the infant gut microbiome for up to three years. The first three years of life represent a critical period for establishing and developing the infant gut microbiome, pivotal in shaping lifelong health outcomes[6,17]. During this time, the infant gut microbiome undergoes dynamic changes, influenced by many factors, including delivery mode, maternal characteristics, infant feeding practices, antibiotic exposure, and environmental factors[17,18,65,66]. These early-life experiences have the potential to profoundly impact microbial colonization patterns and diversity, with far-reaching implications for growth, body composition development, and susceptibility to disease throughout infancy and beyond.

Parity, delivery place, and intrapartum antibiotics may play a key role in microbial colonization patterns of the gut during infancy and early childhood. Parity may influence maternal immune responses, hormonal profiles, and breastfeeding practices, shaping microbial transmission to the neonate during childbirth and breastfeeding[36,37]. Additionally, delivery place and mode may significantly impact initial microbial colonizers encountered by the neonate during childbirth and shape the composition of the gut microbiome[20,23]. Intrapartum antibiotics, while effectively reducing the risk of neonatal GBS infections, may disrupt the maternal and neonatal microbiomes, leading to alterations in microbial colonization patterns in the infant's gut[42,45,67]. Given the implications for health across the lifespan, understanding the influence of early-life exposures on the infant gut microbiome is very important.

Growth trajectories during infancy and early childhood can indicate overall health and development[16,68,69]. Variations in growth patterns, such as deviations from expected weight gain or alterations in body composition, may serve as early indicators of metabolic dysfunction, nutritional deficiencies, or underlying health conditions[24,60,69]. Given that several studies have identified a relationship between the gut microbiome and obesity in humans, it is essential to understand if early-life characteristics of the microbiome are linked to early trajectories of growth in fat mass and lean mass[7,70,71]. Therefore, investigating the association of the microbiome with growth parameters, such as body mass index z-score (BMIz), fat mass index (FMI), and lean mass index (LMI), is essential for identifying potential risk factors for adverse health outcomes to inform early-life intervention strategies.

Moreover, elucidating the interplay between early-life exposures, growth, body composition, and the infant gut microbiome has the potential to uncover microbial patterns contributing to growth and metabolic disorders later in life. Disruptions to the gut microbiome during critical developmental periods may have long-lasting consequences for host metabolism, immune function, and overall health[6,17,65]. This research can inform clinical practice, public health policy, and personalized interventions to promote optimal growth and metabolic health outcomes in children. Interventions aimed at obesity management should be directed at the formative periods of the gut microbiome. The primary outcomes and exposures of interest for this study are depicted in Figure 1.

2.2 Hypothesis

We hypothesize that early-life exposures, including, delivery place, parity, and antibiotic exposure, will significantly influence the composition and diversity of the

infant gut microbiome during the first three years of life. Specifically, we anticipate that home-born infants will exhibit microbial communities enriched with beneficial taxa such as *Bifidobacterium* compared to infants born at the hospital. Moreover, we expect differences in microbial colonization patterns between infants born to nulliparous and multiparous mothers, with multiparous mothers transmitting a more diverse and stable microbial ecosystem to their offspring. Additionally, we anticipate that antibiotic exposure during infancy will lead to alterations in microbial composition and diversity, particularly a decrease in beneficial taxa and an increase in opportunistic pathogens, with potential implications for long-term health outcomes. We hypothesize that the early infant gut microbiota may predict downstream clinical outcomes such as growth (BMIz and D BMIz) and body composition (FMI and LMI) during the first three years of life. It is expected that infants with a rapid growth trajectory (higher Δ BMIz) would show decreased alpha diversity. We hypothesize that specific microbial taxa will be positively correlated with adiposity measures such as FMI, while others will be negatively correlated with lean mass measures such as LMI.

2.3 Research Questions and Aims

The research questions of this project are:

1. Are early-life exposures such as delivery place, maternal parity and intrapartum antibiotics prophylaxis for Group B *Streptococcus* (IAP for GBS) associated with distinct microbial profiles that persist up to three years of life?
2. Is the infant gut microbiome associated with growth and body composition in infants over the first three years of life?

To address these questions, two aims and several sub-aims are proposed:

Aim 1: To investigate how early-life exposures are associated with the gut microbiome from 3 days to 3 years.

a) Determine if gut microbiome alpha and beta diversity are associated with delivery place, maternal parity, and IAP for GBS.
b) Identify bacteria exhibiting differential abundance among infants based on three exposures: delivery place (hospital vs. home), maternal parity (nulliparous vs. multiparous), and antibiotic exposure (no IAP vs. IAP exposure).
c) Assess if any taxa persist over time according to a specific exposure and visualize this pattern in a longitudinal regression plot.

Aim 2: To examine the relationship of the infant gut microbiome with growth and body composition up to 3 years of age.

a) Determine if alpha diversity at 12 weeks, 5 months, 1 year, 2 years, and 3 years relates to BMIz, FMI, and LMI.
b) Determine the association between D BMIz from 12 weeks to 3 years with microbiome alpha diversity at 12 weeks, 5 months, 1 year, 2 years and 3 years.
c) Examine the independent association of gut microbiome beta diversity variation with growth and body composition (BMIz, FMI, LMI) after accounting for perinatal and infant-specific covariates.
d) Visualize clustering patterns of the infant gut microbiome over time and assess if clustering is associated with growth and body composition (BMIz, FMI, LMI).

3.0 Methods

3.1 Sample selection and measurement

The Baby & Mi cohort originally comprises 256 full-term low-risk mother-infant pairs recruited from midwifery practices in Hamilton, Ontario and the surrounding area[72]. Pregnant people were eligible to participate in the study if they were able to communicate in English, under the care of a midwife and planning to give birth vaginally at term[72]. Pre-terms and multiple gestations were excluded from participating. Participants with complete follow-up growth and body composition data from birth to 3 years of age were included in this analysis (n= 245 infant-mother pairs). Data collection timepoints considered for analysis were 3 days, 10 days, 6 weeks, 12 weeks, 5 months, 1 year, 2 years and 3 years[72].

The study design is summarized in Figure 2. Data about the birth were obtained from birth records. At each study timepoint, parents completed a questionnaire and a stool sample was collected[72]. In-person study visits occurred at McMaster Children's Hospital at 12 weeks, 5 months, 1 year, 2 years and 3 years[72]. These sessions involved the submission of stool samples, follow-up data collection, and assessments of infant growth. Anthropometric measures (including body mass index z-score (BMIz), fat mass index (FMI) and lean mass index (LMI)) were conducted at in-person study visits from 12 weeks until 3 years[72]. Recumbent length was measured using a pediatric length board for participants under 2 years of age with precision to the nearest 0.1 cm (Ellard Instrumentation Ltd, WA, USA). Participants that were 2 years of age or older had their height measured with a stadiometer to the nearest 0.1 cm (Ellard Instrumentation Ltd, WA, USA). Body mass index (BMI) was determined by dividing body mass (in kilograms) by the square of length (in meters), and the Z score was computed based on

sex and age-specific standards set by the World Health Organization (WHO)[73]. BMIz scores were calculated at 12 weeks and subsequent timepoints up to 3 years.

A new outcome variable, Δ BMIz, was generated and analyzed with the microbiota at various timepoints (12 weeks, 5 months, 1 year, 2 years, 3 years) to understand how growth 'trajectory' is correlated with the microbiota[68]. Δ BMIz, or Delta BMIz, represents the infant's overall growth trajectory in this study, calculated by taking the difference in BMIz from 12 weeks to 3 years[68]. Body composition was measured at 12 weeks, 5 months and 3 years. At 12 weeks and 5 months of age, weight, fat mass, and lean mass were assessed using an air displacement plethysmography (ADP) system (PEA POD) (Infant Body Composition System Analysis, Cosmed USA, Inc., CA, USA))[72]. At 3 years, fat and lean mass were assessed using dual-energy X-ray absorptiometry (DXA) (Infant Body Composition System Analysis, Cosmed USA, Inc., CA, USA))[72]. The corresponding index was computed as fat mass/ lean mass (in kg) divided by the square of length (in meters)[72].

3.2 Microbiome profiles

Microbial data was collected at all time points via DNA extraction from stool samples[72]. A previously described protocol was modified to maximize the quantitative recovery of bacteria[74–76]. Fresh stool samples were processed within 4 hours, while frozen samples were kept in collection bags in home freezers until retrieval by research staff. Frozen stool samples for 3 days, 10 days, and 6 weeks postpartum were collected at the 12-week follow-up visit[72]. A 1 cm by 1 cm square of the diaper was cut using sterile scissors for fully absorbed diaper samples[72]. Frozen samples were thawed, and DNA was extracted from approximately 0.1 g of stool by mechanical lysis with 2.8 mm

ceramic beads and 0.1 mm glass beads for 3 minutes at 3000 rpm in 800 µL of 200 mM sodium phosphate monobasic (pH 8) and 100 µL guanidinium thiocyanate EDTA N-lauroylsarcosine buffer (50.8 mM guanidine thiocyanate, 100 mM ethylenediaminetetraacetic acid and 34 mM N-lauroylsarcosine)[77,78]. This extract was purified using the MagMAX-96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA) on the MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, CA, USA). A Nanodrop 2000c Spectrophotometer was used to quantify DNA (Thermo Scientific, Mississauga, ON, Canada).

Using barcoded Illumina sequencing, bacterial community profiling of the 16S rRNA gene was done using paired-end reads of the V3 region[72,79]. DNA extraction and amplicon libraries were prepared in the Surette laboratory as described previously[72]. 250 paired-end sequencing was carried out on the MiSeq Illumina sequencer in the Farncombe Genomics Facility (Illumina Inc., San Diego, CA, USA). Illumina sequences were demultiplexed using Illumina's Casava software[42,79]. Barcode, primer, and adapter sequences were trimmed, removing sequences with less than 100 base pairs, using cutadapt[80]. Amplicon sequence variants (ASVs) were inferred using the Divisive Amplicon Denoising Algorithm 2 (DADA2) package in R[81]. Approximately 50,000-100,000 reads per sample were processed using an in-house bioinformatics pipeline[76]. The minimum, mean, and maximum reads per sample are 2804, 83888, and 399123[42,78]. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier against the SILVA v.1.3.8 database; any ASVs not of bacterial origin were excluded[82,83].

Alpha diversity within community diversity is estimated using ASV abundance to determine observed richness and evenness. This was calculated with a depth of 3000

sequences using the phyloseq package in R[84]. Richness refers to the number of species present in a sample, while evenness refers to how evenly the abundance of those species is distributed within the sample[77]. Observed richness and Inverse Simpson were chosen as alpha diversity metrics due to their ability to capture different aspects of microbial community diversity[84]. Observed species is a count of total ASVs in a sample, and Inverse Simpson incorporates both species richness and evenness into its calculation[77,84]. Beta diversity, between community diversity, were calculated using Bray-Curtis dissimilarity on non-rarified relative ASV abundance and then visualized with principal coordinate analysis (PCoA) to determine microbial community structure[77]. Sample-to-sample changes in beta diversity and permutational analysis of variance (PERMANOVA) were calculated using Bray-Curtis dissimilarity at the genus level using the vegan package in R[84,85].

3.3 Covariate assessment

Several variables potentially related to the gut microbiome or clinical measures were prospectively collected. Some baseline variables obtained from birth records include maternal parity, maternal age, maternal BMI, birth delivery place, delivery mode, birth weight, gestational age at birth and sex of the newborn[72]. Maternal parity is a continuous variable defined as the number of times a woman carries a fetus to a viable age[36]. Maternal age is the mother's age at childbirth (calculated from the maternal birthdate and the infant's date of birth). Maternal BMI measures the mother's body fat based on her weight and height before pregnancy[40]. Delivery place refers to where childbirth occurred, such as at home or in a hospital. In contrast, delivery mode indicates

the method of birth, either vaginally or via caesarean section[20]. Gestational age at birth is the length of pregnancy in weeks and days before delivery[72].

Following standard care practices and by recommendation of their healthcare providers, women who screen positive for vaginal Group B *Streptococcus* (GBS) are administered intrapartum antibiotic prophylaxis (IAP)[72]. Data on intrapartum antibiotic usage, including indication, dosage, frequency, and delivery timing, were collected from birth records[72]. IAP indication is the primary categorical variable used for analysis as mothers may have been exposed to IAP for several reasons: C-section, a GBS positive test, infection or a combination of C-section and another indication[72].

Other variables such as infant feeding (breastfeeding or formula), child exposure to antibiotics or probiotics and age at solid food introduction were collected at follow-up study visits – by questionnaire[72]. Based on a prospective report by the parents at each study visit, a categorical feeding variable was created, which categorized participants based on feeding practices until five months as follows: exclusively breastfed, exclusively formula fed or mixed fed[72]. Infants were classified as mixed fed if they consumed formula at least once per week. Additional feeding variables were created to represent mixed feeding duration for infants that were mixed fed up to 3 days, 10 days, 6 weeks, and 12 weeks.

3.4 Statistical analyses

Missing data was handled using generalized estimating equations (GEE), and all analyses were performed using the R statistical software version 4.2.2[86]. Numerical variables were assessed for collinearity using the corrr package in R[87]. Categorical variables were evaluated using directed acyclic graphs (DAGs) using DAGitty

software[88]. All variables, including possible confounders, were assessed for multicollinearity using the variance inflation factor (VIF). Variables with a VIF of 10 or higher were planned to be excluded from the analysis since that would indicate multicollinearity[89]. However, none of the covariates were excluded from the study based on the VIF criteria. All significance tests were done at a 0.05 significance level, assuming a two-sided alternative hypothesis.

The relationship between the microbiome and early-life exposures was first assessed cross-sectionally by determining alpha and beta diversity of the microbiome at all relevant time points (3 days, 10 days, 6 weeks, 12 weeks, 5 months, 1 year, 2 years, 3 years), and then compared it to the exposures of interest- delivery place, maternal parity and IAP for GBS. Linear regression models were run for all exposures and covariates with each alpha diversity metric (Observed richness and Inverse Simpson) at each timepoint[90,91]. An envfit multivariable model was used to determine if the variance in beta diversity of the microbiome is associated with exposures of interest. Envfit is a function in R used to fit environmental factors into an ordination[92]. Due to the compositionality of the microbiome, the data was central log transformed to account for skewed data and be standardized for statistical tests in envfit[93].

The association between bacterial ASV abundance and exposures (delivery place, parity and IAP indication) were assessed using the DESeq2 package in R[94]. DESeq2 uses a negative binomial distribution to model count data and identify differentially expressed genes between experimental conditions or groups[94]. ASV data was collapsed to the genus level based on taxonomy, and a prevalence threshold was set to include taxa present in at least 10% of samples. Only vaginally delivered infants

were analyzed, removing delivery mode as a possible confounder. All timepoints from 3 days to 3 years were assessed using an adjusted p-value of 0.05 for all regression tests[90,91]. Several stratifications were conducted to elucidate microbial profiles for each primary exposure of interest. We selected a subgroup of infants from multiparous mothers who had not been administered IAP to determine the taxa associated with delivery place. For parity, we analyzed the subset of hospital-delivered infants not administered IAP. We selected infants from nulliparous mothers delivered in a hospital setting to test the IAP exposure. Longitudinal models for taxa that persisted across four or more timepoints were constructed using the Lme4 and LmerTest packages in R[91,95]. All longitudinal models were adjusted for interindividual variability.

Growth and body composition were analyzed using the same approach for Aim 1. The outcome measures of interest are BMIz, FMI and LMI. Based on data availability, BMIz was analyzed cross-sectionally at 12 weeks, 5 months, 1 year, 2 years, and 3 years. The alpha diversity of the early microbiota at 12 weeks was compared with BMIz at 1 year, 2 years and 3 years. Growth was analyzed longitudinally by comparing Δ BMIz of each infant with alpha diversity metrics (Observed richness and Inverse Simpson) at all timepoints. FMI and LMI were assessed at 12 weeks, 5 months and 3 years only. Multivariable regression models were run to isolate the specific associations of growth/ body composition with gut microbiome alpha diversity while accounting for other perinatal and infant-specific variables. Variables included are parity, intrapartum antibiotic indication, maternal age at birth, maternal BMI, sex, delivery place, delivery mode, and breastfeeding status. Any significant associations ($p < 0.05$) or trending towards significance ($0.05 < p < 0.1$) were included in the model[90]. Beta diversity was assessed using envfit models and clustering plots for each variable.

4.0 Results

4.1 Early-life exposures are associated with gut microbiome alpha and beta diversity

The Baby & Mi cohort encompasses 245 full-term, low-risk infants exclusively breastfed, with growth and body composition measures documented at intervals from 12 weeks to 3 years of age. A detailed description of the cohort is provided in Table 1. The relationship between delivery mode, vaginal or C-section, and the infant gut microbiome is relatively well characterized; less is known of delivery place, maternal parity, and use of intrapartum antibiotics during labour. Thus, this analysis aimed to determine how early-life exposures are associated with the infant gut microbiome by looking at (1) alpha diversity, (2) beta diversity and (3) bacterial taxonomy. We considered all time points after birth for analysis: 3 days, 10 days, 6 weeks, 12 weeks, 5 months, 1 year, 2 years and 3 years.

Alpha diversity of the infant gut microbiome was first assessed to establish a relationship with exposures of interest: delivery place, parity and IAP for GBS. Figure 3 displays alpha diversity measures, Observed richness and Inverse Simpson, for all infants in the Baby & Mi cohort from 12 weeks to 3 years. Alpha diversity increases over time, marked by a sharp increase from 5 months to 1 year. Figure 4 shows the individual alpha plots for each exposure of interest. All three exposures are significantly associated with Observed richness and Inverse Simpson ($p < 0.05$). Infants born at home to multiparous mothers who were not exposed to IAP during labour had increased alpha diversity in the first three years of life.

Beta diversity analysis offers another way to establish relationships with crucial exposures and understand broader community structure dynamics. Bray-Curtis

dissimilarity distances were utilized in an envfit multivariable model, allowing for a comprehensive exploration of the relationship between various maternal, perinatal, and infant covariates and beta diversity variability across all timepoints (see Figure 5). Parity shows the most significant association with gut microbiome variability from 3 days to 3 years ($p < 0.001$). Similarly, IAP for GBS, delivery place, and delivery mode showed significant associations, most pronounced at 3 days, 10 days, and 6 weeks ($p < 0.01$). This suggests a more immediate association with IAP on microbiome variation during the earliest stages of life. At the same time, factors such as parity and feeding practices exhibit enduring associations for up to 3 years. The feeding variables (d3feeding, d10feeding. w12feeding and m5feeding) classify infants as exclusively breastfed, exclusively formula fed or mixed fed up to a certain timepoint. Significant associations were observed across six timepoints: 3 days, 10 days, 12 weeks, 5 months, 1 year, and 2 years ($p < 0.05$). Although the strongest associations were observed at 5 months and 1 year, the differences remained notable and consistent for up to 2 years. Of particular interest is the observation that delivery place, rather than delivery mode, retained significance at 3 years. The three exposures of interest (delivery place, parity and IAP) are all significantly associated with the alpha and beta diversity of the infant gut microbiome.

4.2 Bacterial gut composition of infants differs based on delivery place, maternal parity and intrapartum antibiotics

We established a relationship between delivery place, parity, and IAP by examining microbiome alpha and beta diversity; now, we focus on identifying bacteria that vary based on these exposures. This cohort was overwhelmingly breastfed, with

only 36 born by C-section. thus, we conducted taxonomic analysis only with infants born vaginally (n= 209). To examine taxonomy, we focused on one exposure at a time while controlling for others, selecting subgroups with sufficient sample sizes. These stratifications are described in Figure 6. The subsequent sub-groups were used to assess the three exposures to determine differentially abundant taxa. To determine differences that persist over time, all timepoints in this cohort were considered for this analysis: 3 days, 10 days, 6 weeks, 12 weeks, 5 months, 1 year, 2 years and 3 years.

4.2.1 Delivery Place

To determine the taxa associated with delivery place (home vs hospital births) while controlling for parity and IAP, we examined taxonomy in the sub-group of infants born to multiparous mothers who were not exposed to IAP (n=127). Differentially abundant taxa associated with hospital vs home births are summarized in Table 2. Taxa associated with delivery place were most predominant at ten days, with more differentially abundant taxa associated with hospital-born infants. This taxonomic association with delivery place contributes 13 differentially abundant taxa across three years. *Bacteroides* were the only taxa that remained more abundant in those born at home – across the first year of life ($p < 0.05$). Figure 7 shows the relative abundance plots of taxa at ten days and two years. Hospital-born infants have more *Escherichia-Shigella* and *Enterococcus* at ten days, while home-born infants have more *Bacteroides.* At two years, hospital-born infants still showed a higher abundance for two bacteria: *Blautia* and *[Ruminococcus] gnavus group* ($p < 0.05$). Since this subgroup has accounted for parity and IAP, it is most likely that these differences in taxonomic abundances are due to the different birth environments. Therefore, amongst vaginally

born babies of multiparous mothers that have been breastfed and have not received IAP, *Enterobacteriaceae*, *Escherichia-Shigella*, *Enterococcus* and *Clostridium* were more often seen at ten days, and this pattern was associated with the hospital birth exposure.

4.2.2 Maternal Parity

To assess the taxa associated with maternal parity, we selected the subgroup of hospital-born infants not exposed to IAP (n=105). As noted in Table 3, 42 differentially abundant taxa are associated with infants with different maternal parity. Taxonomic differences appeared as early as three days and persisted up to two years, with the most variety of taxa observed at one year. Infants of multiparous mothers, 'multips', have a higher population of beneficial taxa at one year, including *Bifidobacterium, Lactococcus, Haemophilus, Erysipelotrichaceae, Dorea, Butyricicoccus* and *Allistipes* ($p < 0.05$). Multips show a higher abundance of *Bifidobacterium* throughout the first year of life (Figure 8a). Figure 8b shows a longitudinal effect plot of parity and age on *Bifidobacterium* relative abundance during the first year of life. Parity is significantly associated with *Bifidobacterium* relative abundance ($p= 0.0008$). The model accounts for interindividual variability of each sample. The infant's age does not independently influence the variation in *Bifidobacterium* relative abundance. However, when considered in conjunction with parity, a statistically significant combined effect is observed ($p= 0.03$). Multiparous mothers' infants exhibit higher levels of *Bifidobacterium* in early life, although it gradually declines over time and reaches a similar abundance in infants born from nulliparous mothers. Nulliparous infants, 'nullips', had higher abundances of *f__Clostridiaceae* at 6 and 12 weeks only ($p < 0.05$). Therefore, amongst vaginally delivered hospital-born babies that have been breastfed

and have not received IAP, *Bifidobacterium* was more often seen at all timepoints up to 1 year, and this pattern was associated with maternal parity.

4.2.3 Intrapartum Antibiotic Prophylaxis

The subgroup of nulliparous hospital-born infants was selected to assess the taxa associated with IAP for GBS (n= 53). Nullips were chosen since this group had the closest even distribution between infants with no IAP (n= 35) and those exposed to IAP (n=18). IAP for GBS showed the most significant and prolonged association with taxonomic variation across the first three years of life. Table 4 shows 81 differentially abundant taxa observed across all timepoints for infants with differing IAP exposures. Taxa that persisted across various timepoints in infants not exposed to IAP are *Bacteroides, Bifidobacterium, Streptococcus* and *Escherichia-Shigella* ($p < 0.05$). Infants exposed to IAP showed a higher abundance of *Clostridium* and *Enterococcus* at 12 weeks and 5 months, respectively ($p < 0.05$). The relative abundance of *Bifidobacterium* is reduced in individuals administered IAP (Figure 9a). Figure 9b shows that generally, *Bifidobacterium* decreases over time for both groups. The difference in *Bifidobacterium* relative abundance is significantly associated with infant age ($p= 0.00007$) and not by intrapartum antibiotics ($p > 0.05$). While at a much lower relative abundance, *Streptococcus* was elevated in infants with IAP during the first three years of life (Figure 10a). Similarly to *Bifidobacterium*, *Streptococcus* decreases over time; however, this trend is primarily associated with infant age ($p= 0.00007$) and not IAP ($p > 0.05$) (Figure 10b). This signifies that while *Bifidobacterium* and *Streptococcus* are differentially abundant between infants with/ without IAP at specific timepoints, this trend over time is primarily associated with the infant's age. Thus,

amongst vaginally delivered hospital-born babies of nulliparous mothers that have been breastfed, more *Bifidobacterium* and less *Streptococcus* is observed up to 3 years, and this pattern was associated with the IAP for GBS exposure.

4.3 Growth and body composition are associated with alpha diversity at five months

Alpha diversity of the infant gut microbiome was used to establish a relationship with clinical outcomes: Body mass index z score (BMIz), Fat mass index (FMI) and Lean mass index (LMI). The number of infants with growth and body composition metrics at each timepoint is summarized in Table 5. Most infants had follow-up BMIz data, while a good portion of body composition data was lost at follow-up timepoints. At 12 weeks, 5 months and 3 years infants without either FMI or LMI metrics were 36, 75 and 137, respectively. At 5 months, infants that weighed more than 8 kg could not adequately fit in the PEAPOD machine; thus, the body composition of several infants could not be accurately assessed. Across all time points, BMIz, FMI and LMI distributions conform to a standard Gaussian pattern, with only a handful of infants positioned near the lower and upper ends of the distribution (see Figure 11). Such distributions were expected, considering the low-risk status, breastfeeding practices, and predominance of vaginal deliveries among infants enrolled in the Baby & Mi study.

We adopted a cross-sectional approach to explore how alpha diversity at each timepoint correlates with BMIz scores, aiming to understand the microbiome's relationship with growth. Figure 12 illustrates critical timepoints that show associations with alpha diversity metrics. BMIz was related to Observed richness but not Inverse Simpson at 5 months ($p < 0.05$). We saw no relationship between alpha diversity measures and BMIz at 12 weeks, 1 year, 2 years and 3 years ($p > 0.1$). When testing

alpha diversity associated with BMIz in a multivariable model with covariates (parity, intrapartum antibiotic indication, maternal age at birth, maternal BMI, sex, delivery place, delivery mode, and breastfeeding status), an independent association was noted for BMIz and Inverse Simpson at five months ($p < 0.05$).

We also performed a longitudinal analysis by comparing alpha diversity of the 12-week microbiome and BMIz for 153 infants at 3 years, and no significant association was observed (data not shown, $p > 0.1$). The 12-week microbiome also doesn't correlate with BMIz at 1 year and 2 years. Calculated as the difference in BMIz from 12 weeks to 3 years, Δ BMIz provides an overview of the changes in the weight for height profile (i.e. body size) that deviate from the usual growth. Due to the relatively uniform distribution of BMIz among infants, it was not feasible to categorize them into distinct growth trajectories based on Δ BMIz. No statistically significant association exists between Δ BMIz and alpha diversity at any timepoint (data not shown, $p > 0.1$). While significant associations were found at 5 months, no consistent trend in alpha diversity with BMIz emerged across all timepoints.

The same approach for BMIz was adopted to assess the relationship between body composition metrics (FMI and LMI) and alpha diversity. Figure 13 illustrates the key timepoints that showed associations between alpha diversity measures and body composition. FMI is associated with Observed richness but not Inverse Simpson at 12 weeks ($p < 0.05$). FMI was only associated with Inverse Simpson at five months ($p < 0.05$). We saw no relationship between alpha diversity and FMI at 1 year, 2 years and 3 years ($p > 0.05$). LMI is associated with Observed richness at 5 months and Inverse Simpson at 3 years ($p < 0.05$). We saw no relationship between alpha diversity measures

and LMI at 12 weeks, 1 year, and 2 years ($p > 0.05$). While there is a significant association of alpha diversity with FMI and LMI at 5 months, the 12-week microbiota doesn't show trends with body composition measures at 1 year, 2 years or 3 years. We tested alpha diversity associated with FMI and LMI in two separate multivariable models with the same covariates tested for BMIz. Associations observed for FMI at 12 weeks and LMI at 3 years were no longer statistically significant (data not shown, $p > 0.1$). At 5 months, the associations for FMI and LMI still hold when accounting for additional covariates. FMI is significantly associated with Inverse Simpson ($p < 0.05$), and LMI is significantly associated with Observed Richness ($p < 0.01$). Overall, infants with lower BMIz, FMI, and LMI measures show greater alpha diversity at 5 months of age, and at no other timepoint.

4.4 Beta diversity of the infant gut microbiome shows limited associations with growth and body composition

The primary outcome measure, BMIz, was not independently associated with beta diversity of the gut microbiome at any timepoint (see Figure 5). However, when assessed with only vaginally delivered infants, BMIz was significantly associated with beta diversity at 5 months ($p= 0.032$, data not shown). Due to statistical considerations, individual envfit models were necessary for the other two outcome variables— FMI, and LMI—ensuring precise examination of their associations with beta diversity. Combining all outcome variables into one model could introduce confounding factors and obscure the unique relationships between beta diversity and each growth/ body composition measure. At 12 weeks, beta diversity of the microbiome was significantly associated with FMI ($p < 0.05$, see Figure 14). LMI was not associated with beta

diversity at any timepoint ($p > 0.05$, data not shown). The only body composition measure associated with beta diversity was FMI at 12 weeks. The primary outcomes (BMIz, FMI and LMI) showed limited associations with gut microbiome beta diversity. FMI was associated with beta diversity of all infants at 12 weeks (n=220), and BMIz was only associated with vaginally delivered infants at 5 months (n=189).

Another method for examining associations between beta diversity and the primary outcome variables was by looking at beta diversity clustering patterns in a PCoA plot. Figure 15 illustrates the overall community structure using Bray-Curtis dissimilarity metrics at each timepoint. At 12 weeks and 5 months, similar communities are observed with minor variations, reflecting the infant gut's ongoing maturation and rapid colonization. A significant shift occurs at 1 year, marked by more clustered microbiomes coinciding with the cessation of breastfeeding and introduction of solid food. Despite overlaying BMIz scores, no significant clustering is observed at any timepoint ($p > 0.05$). Additionally, no statistically significant clustering was observed for FMI and LMI (data not shown). Consequently, exploration into taxonomy was not pursued due to the lack of strong associations between beta diversity and outcome variables (BMIz, LMI, and FMI). While associations were observed for BMIz at 5 months and FMI at 12 weeks, we didn’t observe any significant patterns across all time points. Overall, there is a minimal relationship between alpha and beta diversity of the gut microbiome and BMIz, FMI, and LMI from 12 weeks to 3 years of age.

5.0 Discussion

5.1 AIM 1: Early-life factors are predictors of gut microbiome variation

The connection between delivery place, maternal parity and intrapartum antibiotics has been highlighted in our research as being significantly associated with infant gut microbiome alpha and beta diversity. Elevated microbial diversity observed in home-birth infants of multiparous mothers not exposed to IAP, highlights the significance of birth environment and maternal factors in shaping early microbial colonization. The early associations observed for delivery place, parity and IAP for GBS on microbiome composition underscore the complex interplay that environmental, maternal, and medical intervention factors may play in establishing the infant gut microbiome.

Infants born at home have a unique microbial profile, with higher levels of *Bacteroides*, indicating potential health benefits from less medicalized environments in early life[21–23]. The number of previous pregnancies the mother has had is associated with increased microbial diversity, especially beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, in multips, suggesting that repeated pregnancies may provide microbial advantages[36,37,40]. Exposure to intrapartum antibiotic prophylaxis showed long-lasting disruption in microbial diversity, characterized by a decrease in beneficial bacteria (i.e. *Bifidobacterium*) and an increase in potentially harmful bacteria (i.e. *Streptococcus*). The place of birth, maternal parity, and intrapartum antibiotics show strong associations with the infant gut microbiome during the first three years of life.

5.1.1 Delivery Place

Taxonomic analysis revealed distinct microbial profiles for infants born in hospitals compared to those born at home. This finding is especially noteworthy when considering the presence of *Bacteroides* in home births, suggesting the possibility of more advantageous or protective microbial colonization in less medically intensive environments[20,21,24,96]. The *Bacteroides* genus is linked to various health benefits, such as supporting the immune system and establishing a strong gut barrier function, indicating that home births may provide a more favourable environment for beneficial microbial colonization[24,97]. This observation is consistent with studies that have examined the impact of the environment on microbial colonization and the potential health implications in home birth settings with less medical intervention[24,96].

From as early as ten days after birth, the presence of *Bacteroides* in infants born at home and the prevalence of *Escherichia-Shigella and Enterococcus* in infants born in hospitals highlight the potential influence of the birth environment on microbial colonization[23,96]. The early high dominance of *Enterococcus* in hospital-born infants may indicate the limited microbial diversity typically associated with hospital-acquired infections[21,23,96]. While not all species are opportunistic, an elevated level of *Enterococcus* as early as ten days might suggest a disrupted gut microbiome. Few studies have examined the impact of birth environments on the infant gut microbiome due to disproportionate sample sizes of home and hospital-delivered infants. In this study, midwives facilitated delivery, allowing sufficient home-born infants to statistically compare with those delivered in the hospital[72].

Our findings present a new perspective on how different birth environments can support unique microbial communities from a young age, potentially impacting immune development and disease susceptibility in the long run[23,25]. Most associations between delivery place and microbial colonization were noticeable at ten days, indicating that differences in early environmental exposures may have a more significant association with initial microbial diversity rather than long-term microbial makeup. Additional factors like parity, breastfeeding, and antibiotic exposure may then be more strongly associated with gut colonization as the infant ages.

5.1.2 Maternal Parity

Our study demonstrates that maternal parity is strongly associated with the infant gut microbiome. Gut microbial diversity is increased in infants born to mothers with high parity. This provides important insights into the dynamics of microbial transmission, as it has been previously reported that maternal factors may enrich the infant's microbial environment[37,40]. The link between parity and the presence of beneficial bacteria such as *Bifidobacterium* in the first year of life implies that higher parity may confer health benefits to subsequent offspring[36]. Infants born to multiparous mothers had a higher abundance of *Bifidobacterium*, a genus known for promoting gut health and immune development by breaking down complex carbohydrates, providing essential vitamins, and inhibiting the growth of pathogenic bacteria[6,27,36]. The elevated levels of *Bifidobacterium* in these infants suggest a more favourable gut environment, which may contribute to improved immune function and overall health[98].

Ten days after birth, infants of multiparous moms had higher numbers of *Lactobacillus*, which is associated with positive health outcomes such as improved

immune function and reduced risk of infection[99]. Additionally, infants born to multiparous mothers showed a higher presence of *Bacteroides* three months after birth. This genus plays a crucial role in the breakdown of complex molecules, the development of the immune system, the maintenance of gut health and the prevention of inflammatory diseases[27,36,100]. A higher abundance of *Bacteroides* suggests that the offspring of multiparous mothers may have a more beneficial gut microbial community, which may improve health outcomes[36,37]. *Clostridium* was higher in infants born to mothers who had never given birth before (i.e. nulliparous). This increased bacterial presence at six months postpartum may indicate immaturity or imbalance of the gut microbiome, potentially exacerbating gastrointestinal infection[98]. These results emphasize the importance of maternal factors in potentially influencing the gut microbiota of infants[36].

Although parity is associated with infant gut microbiome alpha diversity, beta diversity and taxonomy, the exact mechanism by which this occurs is unknown and is beyond the scope of this research. However, several possible explanations exist for the associations between parity and the infant gut microbiome. One possibility is the vertical transfer of bacteria from the mother's microbiota during delivery and postpartum interactions[36]. Multiparous mothers may have an established and highly diverse microbiota in early pregnancy, which can be transmitted to their infants through breastfeeding and close physical contact, resulting in a diverse gut microbiota[37,40]. Another possibility is the "sibling effect," where older children in higher parity households introduce a wider variety of microbes to the newborn through close physical interactions, further diversifying and enriching the infant's gut microbiome[101]. This

environmental exposure may work by complementing vertical transfer from the mother, offering additional pathways for beneficial microbial colonization.

Overall, this research showed that infants born to multiparous mothers have a taxonomic profile that coincides with more potentially beneficial bacteria, such as *Lactobacillus*, *Bacteroides*, and *Bifidobacterium*, necessary for adequate early-life gut microbiome development [5,29]. While most studies have examined associations between parity and the maternal microbiome, none have examined how parity, as a maternal factor, may impact the infant gut microbiome. The maternal gut microbiome needs to be explored in conjunction with the infant gut microbiome to get the full picture of the interplay between maternal health, parity and microbial gut diversity.

5.1.3 Intrapartum Antibiotic Prophylaxis

In our study, intrapartum antibiotic prophylaxis (IAP) for Group B *Streptococcus* (GBS) exhibited the most persistent association with taxonomic variation in the infant gut microbiota across the first three years of life. Our analysis revealed 81 differentially abundant taxa associated with the IAP exposure, indicating a substantial disruption in microbial colonization[49,72]. This disruption was characterized by higher abundances of *Clostridium* and *Enterococcus* in IAP-exposed infants. At the same time, beneficial commensals like *Bifidobacterium* and *Bacteroides* were notably reduced or absent at various time points[41,42,72].

The absence of critical commensal taxa, particularly *Bifidobacterium*, in infants exposed to IAP suggests a compromised gut ecosystem that may facilitate the proliferation of harmful microbes[42,47,48,67]. Interestingly, longitudinal analysis revealed a gradual decrease in *Bifidobacterium* abundance over time for both IAP-exposed and

non-exposed infants. This trajectory of *Bifidobacterium* during the first three years is associated with the infant's age rather than antibiotic exposure. Although *Streptococcus* was elevated in infants with IAP, its abundance decreased over time due to age-related factors rather than antibiotic exposure. This aligns with previous findings showing that IAP initially disrupts gut colonization in early life, but its effects diminish over time as other factors influence microbiome development[42]. While some taxa remained differentially abundant for up to three years, our analysis suggests that factors other than IAP may influence gut bacterial diversity during the first three years of life.

The association of IAP with a reduction in *Bifidobacterium* and an increase in *Streptococcus* underscores the complicated association of antibiotics on the developing gut microbiome[45,67]. These shifts in microbial composition may have lasting implications for immune development, metabolic health, and disease susceptibility, aligning with concerns raised by previous studies[42,47,49,102] The persistence of taxa differences associated with IAP across the first three years of life suggests that the potential impacts of early antibiotic exposure are not only immediate but also enduring, warranting careful consideration in using IAP[41,42,45]. The effectiveness of IAP in preventing infection is clear however interventions could be implemented to avoid excessive perturbation of the early gut microbiomes of low-risk infants. Rapid testing of Group B *Streptococcus* during labour could be implemented so that only individuals who are at high risk of infection receive IAP, ensuring that the microbiome of low-risk infants is protected from unnecessary antibiotic disruption[41,42].

5.2 AIM 2: Growth and body composition measures show minimal associations with the infant gut microbiome

This study has examined the relationships between alpha and beta diversity of the infant gut microbiome and growth and body composition up to three years of age. Our findings reveal a significant association between alpha diversity and BMIz at five months but not at later time points, suggesting a critical window during which the gut microbiome may be associated with infant growth development[17]. The association with observed richness, rather than evenness, indicates that a diverse array of microbial species is potentially more crucial for influencing growth during this early stage[16,24]. Early microbial diversity can have lasting implications on health outcomes, such as immune development and metabolic health[5,65,68]

The association of alpha diversity with FMI and LMI at five months may indicate differential impacts of the microbiome on fat and lean mass accumulation. These context-dependent associations further underscore the complex role of the microbiome in possibly influencing body composition and metabolic health[15,103,104]. While only observed at one timepoint, studies have indicated that the gut microbiota can affect energy harvest from the diet and influence fat storage and metabolism[70,105]. For instance, it has been demonstrated that differences in gut microbiota composition could lead to variations in energy extraction from the diet, thereby influencing fat mass[70]. These differential influences on fat and lean mass suggest that specific microbial taxa may have distinct roles in metabolic processes that govern body composition, highlighting the necessity for a detailed understanding of these relationships. However, the association of microbial alpha diversity with BMIz, FMI and LMI at no additional

timepoints may also suggest that another underlying factor may be confounding these findings. The introduction of solid food and breastfeeding occurs around 5 months and are known to foster colonization of microbes best suited for assisting in fibre consumption[77]. The lack of associations across 3 years suggests that the outcome variables are not independently associated with alpha diversity, thus, the boom of microbial diversity observed at 5 months may be confounded by solid food introduction and breastfeeding.

FMI was the only outcome variable significantly associated with beta diversity at 12 weeks, suggesting that the early infant gut microbiome may be more associated with adiposity development than lean mass. The lack of significant associations between primary outcome measures (BMIz, FMI, LMI) and beta diversity across all timepoints suggests that additional, unmeasured factors may influence the relationship between the microbiome and growth outcomes. This observation highlights the need for comprehensive models incorporating various influences, including diet, antibiotic exposure, and other environmental factors, to understand the microbiome's role in early development fully.

Studies have found associations between the microbiome and growth trajectory in populations with very drastic growth developments or levels of malnutrition[16,60,68,69]. Our study could not confirm these longitudinal findings due to the lack of variability of infants with similar BMIz, FMI and LMI across all timepoints in our cohort. All associations observed were cross-sectional, and we could not determine if the gut microbiome is associated with growth trajectories over time. Cross-sectional observations only show a ‘snapshot in time’ and cannot establish causality or the

directionality of associations. Longitudinal studies are essential to understanding the dynamic interactions between the infant gut microbiome, growth and body composition to elucidate the temporal sequence of these relationships[17,66,68].

5.3 Strengths and Limitations

This study has various strengths in both study design and data collection. Data was collected longitudinally at various timepoints from birth to three years which allowed us to canvas the crucial developmental phase of gut microbiome maturation. The cohort has a large sample size with a high rate of follow-up even at the 3-year mark. Infants were low risk had low interventions and high rates of breastfeeding which allowed us to look at various early-life exposures and body composition outcomes in a homogenous healthy population. Labor and delivery were assisted by the midwives at McMaster University which allowed for a higher-than-normal home birth population for a cohort of this size. There was also enough variation of mothers with differing parities which allowed us to analyze the association of the infant gut microbiome and two underexplored exposures- delivery place and maternal parity.

While our study provides valuable insights into the early-life factors influencing the infant gut microbiome, several limitations must be acknowledged. Firstly, the study's observational design limits the ability to infer causation from the observed associations between delivery place, parity, IAP for GBS and microbial colonization patterns. Additionally, our reliance on 16S rRNA gene sequencing, while helpful in identifying bacterial taxa at the genus level, does not capture the functional potential of the microbiome or detect non-bacterial microbes, which could play significant roles in early gut colonization. The lack of accurate taxonomic classification at the species level

also limits the ability to draw conclusions on the benefits of infants that confer specific microbial profiles. The homogeneity of our cohort—comprising full-term, low-risk infants predominantly from vaginal deliveries—limits the applicability of our findings to broader, more diverse populations. Although sufficient to detect associations, our sample size may not be large enough to generalize the findings to different populations. Our study population may be representative of only some demographic groups, limiting the applicability of our findings to different ethnicities, socioeconomic statuses, and geographic regions.

The potential for unmeasured factors, such as maternal diet, maternal gut microbiome, environmental exposures, and genetic influences, may confound results observed for early-life exposures, growth, and body composition. Variations in sample collection, storage, and DNA extraction methods could introduce biases despite our efforts to standardize these procedures. The cross-sectional nature of some aspects of our data analysis does not capture temporal changes in the microbiome. It limits our understanding of how dynamic shifts in microbial communities correlate with growth and development. The lack of detailed clinical and health data beyond the first three years restricts our ability to link early microbiome patterns with long-term health outcomes in older children.

5.4 Future Directions

Building upon this study's findings, several approaches can be taken to uncover additional relationships between early-life exposures, growth and the infant gut microbiome. Extending the follow-up period beyond the first three years of life could provide deeper insights into the long-term impacts of early microbial colonization on

growth trajectories and developmental milestones throughout childhood. Investigating how early microbial exposures influence the microbiome and health into later childhood and adolescence would help to identify critical windows of microbial influence and inform potential interventions.

While this study established associations between microbial profiles and early-life factors such as delivery place, parity, and IAP for GBS, future research should uncover the underlying mechanisms driving these relationships. Functional microbiome analyses, including metagenomic and metabolomic approaches, could shed light on the specific microbial functions and metabolic pathways influenced by these early-life exposures. Future research should explore the impact of probiotics, dietary modifications, and other interventions to promote similar microbial profiles in these infants, ultimately improving their health outcomes. Additionally, interventional studies designed to modify early-life exposures, such as maternal diet during pregnancy, probiotic supplementation, or controlled antibiotic use, could test the causality of observed associations and offer potential strategies for optimizing the infant gut microbiome. Evaluating the efficacy and safety of such interventions in promoting beneficial microbial colonization and improving health outcomes would be a valuable next step.

Expanding this research to include larger, more diverse populations and settings is crucial to understanding the generalizability of the findings. Investigating how cultural, dietary, and environmental differences influence the infant gut microbiome across various geographic and socioeconomic contexts can provide a more comprehensive understanding of the factors shaping microbial development and health.

Furthermore, integrative approaches combining microbial, genetic, immunological, and environmental data could enhance our understanding of the complex interplay between the gut microbiome and host physiology. Multi-omics studies and systems biology approaches could provide a holistic view of how early-life exposures influence microbial colonization and subsequent health outcomes.

Translating research findings into clinical practice represents a critical future direction. Developing guidelines and recommendations for healthcare providers on optimizing early-life microbial exposures, including delivery practices, antibiotic use, and feeding practices, could help promote healthy microbial development and prevent adverse health outcomes. By pursuing these future directions, researchers can continue to unravel the complexities of the infant gut microbiome and its critical role in health and disease. These efforts will contribute to developing evidence-based strategies for optimizing early-life microbial exposures, ultimately improving health trajectories from infancy through adulthood.

6.0 Conclusion

This thesis explored the associations of early-life factors—namely, delivery place, maternal parity, and intrapartum antibiotics—and growth and body composition measures on the infant gut microbiome over the first three years of life. Utilizing the extensive data from the Baby & Mi cohort study, this investigation sheds light on the complex interplay between these key variables and their collective associations with the gut microbiome, underlining their potential implications for the trajectory of infant health and development.

Early-life exposures showed the most pronounced associations with infant gut microbiome diversity and bacterial taxonomy. A significant association between delivery place and the infant gut microbiome composition was determined in early life, with distinct microbiomes enriched with *Bacteroides* observed in infants delivered at home. The most compelling insight is the relationship between the infant microbiome and maternal parity. Our research indicates that infants of multiparous mothers show increased diversity and the enrichment of *Bifidobacterium* during the first year of life. This may hint at a possible advantage that women with higher parity might confer on the infant gut microbiome, suggesting a legacy effect of maternal parity on the infant's health potential. While parity remains understudied, these findings suggest that maternal history must not be overlooked when considering factors influencing infant gut microbiome development. The proliferation of taxa such as *Streptococcus* and *Clostridium* in infants exposed to IAP for GBS warrants careful consideration from physicians to avoid unnecessary perturbation of the infant’s gut with excessive antibiotic exposure. Microbiome diversity was only associated with BMIz, FMI, and

LMI at 5 months. Since no longitudinal patterns were observed, it can be concluded that the microbiome is not associated with growth and body composition trajectories in this population of low-risk full-term infants who were predominantly exclusively breastfed and vaginally delivered.

In conclusion, this research reveals new insights into the relationship between early-life exposures and the infant gut microbiome during the critical first three years of life. While causal relationships with the infant gut microbiome remain unknown, factors such as maternal parity and delivery place warrant further exploration as possible early-life predictors of childhood health. By identifying these associations between delivery place, parity, IAP for GBS, and the infant gut microbiome, we have also established a framework for future research to develop clinical interventions aimed at optimizing early-life exposures to improve infant health and development.

7.0 Figures and Tables

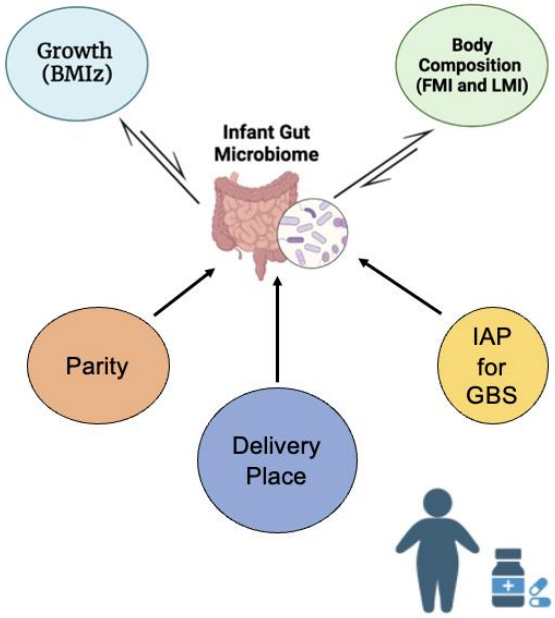


Figure 1: Primary Outcomes and Exposures. Schematic showing the Primary Outcomes and Exposures of interest studied in tandem with the infant gut microbiome. To answer the first aim, microbiome diversity and taxonomy are treated as the main outcome variables when assessing early-life predictors as exposures of interest: delivery place, maternal parity and intrapartum antibiotics for Group B *Streptococcus* (IAP for GBS). In the second part of the analysis, the early infant gut microbiome, specifically microbial alpha and beta diversity, is treated as the primary exposure of interest, with growth (BMIz) and body composition (FMI and LMI) as the primary outcome variables. Figure created on Biorender.com.

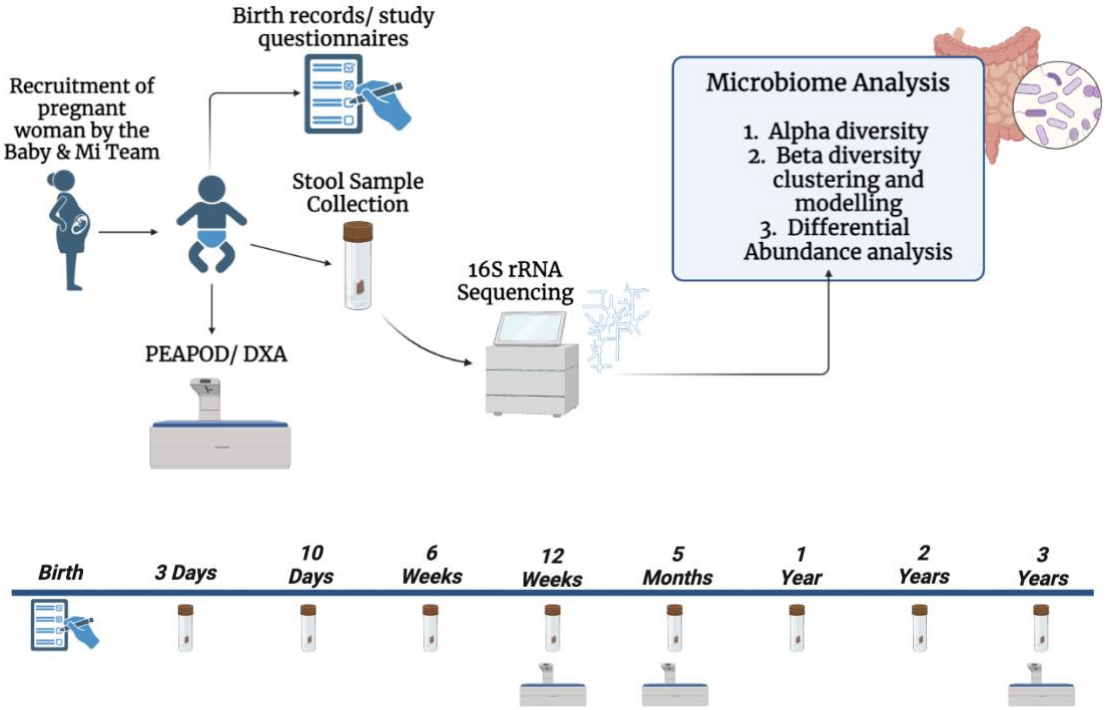


Figure 2: Sample Collection and Study Design. Schematic showing the Study Design pipeline for this analysis investigating the association of the Infant Gut Microbiome with Early-Life Exposures, Growth and Body Composition. Timeline for data collection of stool samples and body composition metrics from PEAPOD/ DXA are not drawn to scale. Figure created on Biorender.com.

Table 1: Description of the cohort. The table shows the characteristics and exposures of the cohort, stratified by delivery mode and exposure to intrapartum antibiotics. Values shown are count (%) for binary and categorical variables or median (inter-quartile range) for continuous variables.

	Vaginal birth		**Caesarean section**
	Not exposed to IAP	**Exposed to IAP**	
	n=170 n (%) or median (IQR)	n= 40 n (%) or median (IQR)	n=33 n (%) or median (IQR)
Maternal pre-pregnancy BMI (kg/m^2)	24.05 (21.13, 25.84)	23.31 (21.32, 26.10)	23.81 (22.14, 26.76)
Missing	1	0	0
Gestational weight gain (kg)[1]	13.7 (10.9, 16.4)	16.2 (12.7, 19.5)	16.3 (13.1, 20.0)
Missing	22	2	3
Maternal age at birth (years)	31.6 (30.5, 34.8)	31.9 (30.0, 33.4)	32.8 (30.1, 33.8)
Missing	1	0	0
Parity			
0	43 (25.3)	22 (55.0)	22 (66.7)
1	84 (49.4)	15 (37.5)	9 (27.3)
2	30 (17.6)	2 (5.0)	2 (6.1)
3	9 (5.3)	1 (2.5)	0 (0)
4	3 (1.8)	0 (0)	0 (0)
5	1 (0.6)	0 (0)	0 (0)
Ethnicity			
Asian	2 (1.4)	0 (0)	0 (0)
Black	0 (0)	1 (3.0)	0 (0)
Hispanic	0 (0)	0 (0)	1 (3.7)
White	127 (90.7)	31 (93.9)	25 (92.6)
Other	11 (7.9)	1 (3.0)	1 (3.7)
Missing	30	7	6
GBS status			
Positive[2]	16 (9.4)	35 (87.5)	4 (12.1)
Negative	135 (79.4)	2 (5.0)	28 (84.8)
Not screened	17 (10.0)	3 (7.5)	1 (3.0)
Inconclusive	2 (1.2)	0 (0)	0 (0)
Gestational age at birth (days)	281 (277, 286)	279 (272, 285)	282 (276, 287)

Home birth	65 (38.2)	8 (20.0)	0 (0)
Type of intrapartum antibiotic			
Penicillin G	-	36 (90.0)	5 (15.2)
Cefazolin	-	0	22 (66.7)
Clindamycin	-	1 (2.5)	0
Ampicillin	-	1 (2.5)	2 (6.1)
Gentamicin	-	1 (2.5)	1 (3.0)
Cephalexin	-	0	1 (3.0)
Ceftriaxone	-	1 (2.5)	1 (3.0)
Metronidazole	-	0	1 (3.0)
Unknown	-	1 (2.5)	6 (18.2)
Intrapartum antibiotics indication			
Caesarean section	-	-	25 (75.8)
GBS prophylaxis	-	38 (95.0)	1 (3.0)
Signs and symptoms of infection	-	2 (5.0)	1 (3.0)
Caesarean section and another indication	-	0 (0)	6 (18.2)
IAP duration (hours)	-	16.0 (6.2, 238.0)	4.5 (0.8, 42.0)
Missing	-	2	9
Intrapartum antibiotics for ≥ 4 hours	-	25 (65.8)	4 (16.7)
Missing	-	2	9
Birth Sex			
Female	88 (51.8)	17 (42.5)	14 (42.4)
Male	82 (48.2)	23 (57.5)	19 (57.6)
Birth weight (g)	3600 (3345, 3905)	3543 (3296, 3916)	3654 (3320, 4070)
Breastfeeding			
At 12 weeks	165 (98.8)	38 (97.4)	27 (90.0)
Missing	3	1	3
At 5 months	155 (95.7)	38 (95.0)	24 (80.0)
Missing	8	0	3
At 1 year	123 (74.5)	29 (74.4)	22 (68.8)
Missing	5	1	1
Age at weaning (months)	15.0 (11.5, 24.0)	15.0 (11.0, 26.0)	15.5 (12.5, 20.5)
Did not breastfeed	0 (0)	0 (0)	1 (3.3)
Not weaned by 3 years	13 (9.2)	3 (8.8)	1 (3.3)
Missing	28	6	3
Introduction of formula or other milk at ≤ 5 months	54 (32.7)	19 (47.5)	19 (61.3)
Missing	5	0	2
Introduction of food at ≤ 5 months	42 (25.8)	15 (37.5)	12 (38.7)
Missing	7	0	2
Infant oral or IV antibiotic use			

Birth to 10 days	4 (2.4)	3 (7.5)	0 (0)
Missing	2	0	2
10 days to 6 weeks	0 (0)	2 (5.0)	0 (0)
Missing	2	0	1
6 weeks to 12 weeks	1 (0.6)	0 (0)	0 (0)
Missing	3	1	2
12 weeks to 5 months	1 (0.6)	0 (0)	0 (0)
Missing	8	0	2
5 months to 1 year	31 (18.9)	8 (21.1)	5 (16.1)
Missing	6	2	2
1 year to 2 years	49 (33.1)	11 (30.6)	18 (62.1)
Missing	22	4	4
2 years to 3 years	31 (21.8)	10 (30.3)	12 (40.0)
Missing	28	7	3

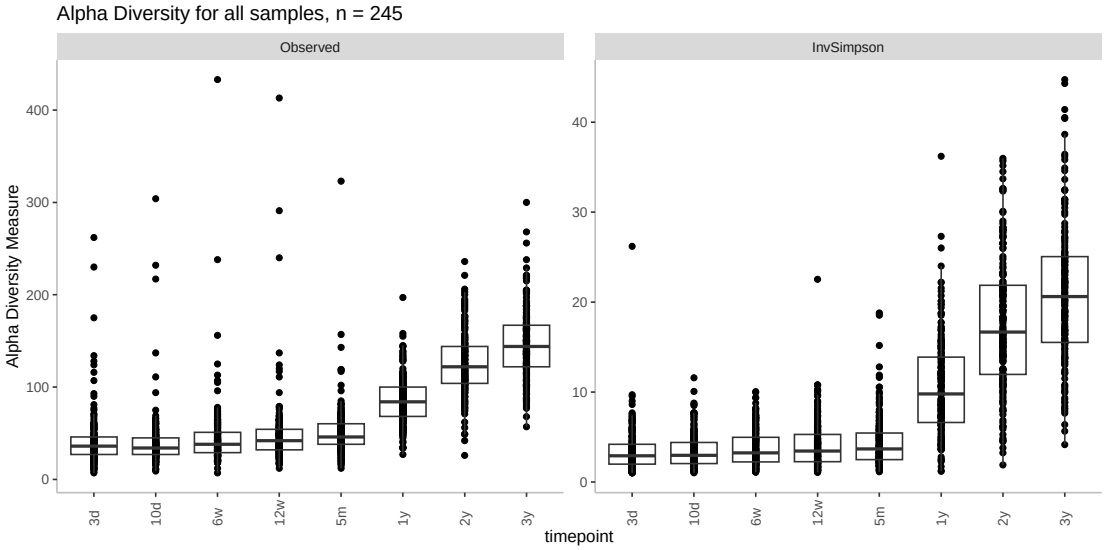


Figure 3: Alpha diversity increases in the first three years of life. Boxplots of alpha diversity value distribution for both indices (Observed richness and Inverse Simpson) of all infants in the Baby & Mi cohort. The line inside each box represents the median value, and outliers are shown as dots. The general trend is an increase in alpha diversity from 3 days to 3 years, with the sharpest increase observed between 5 months and 1 year.

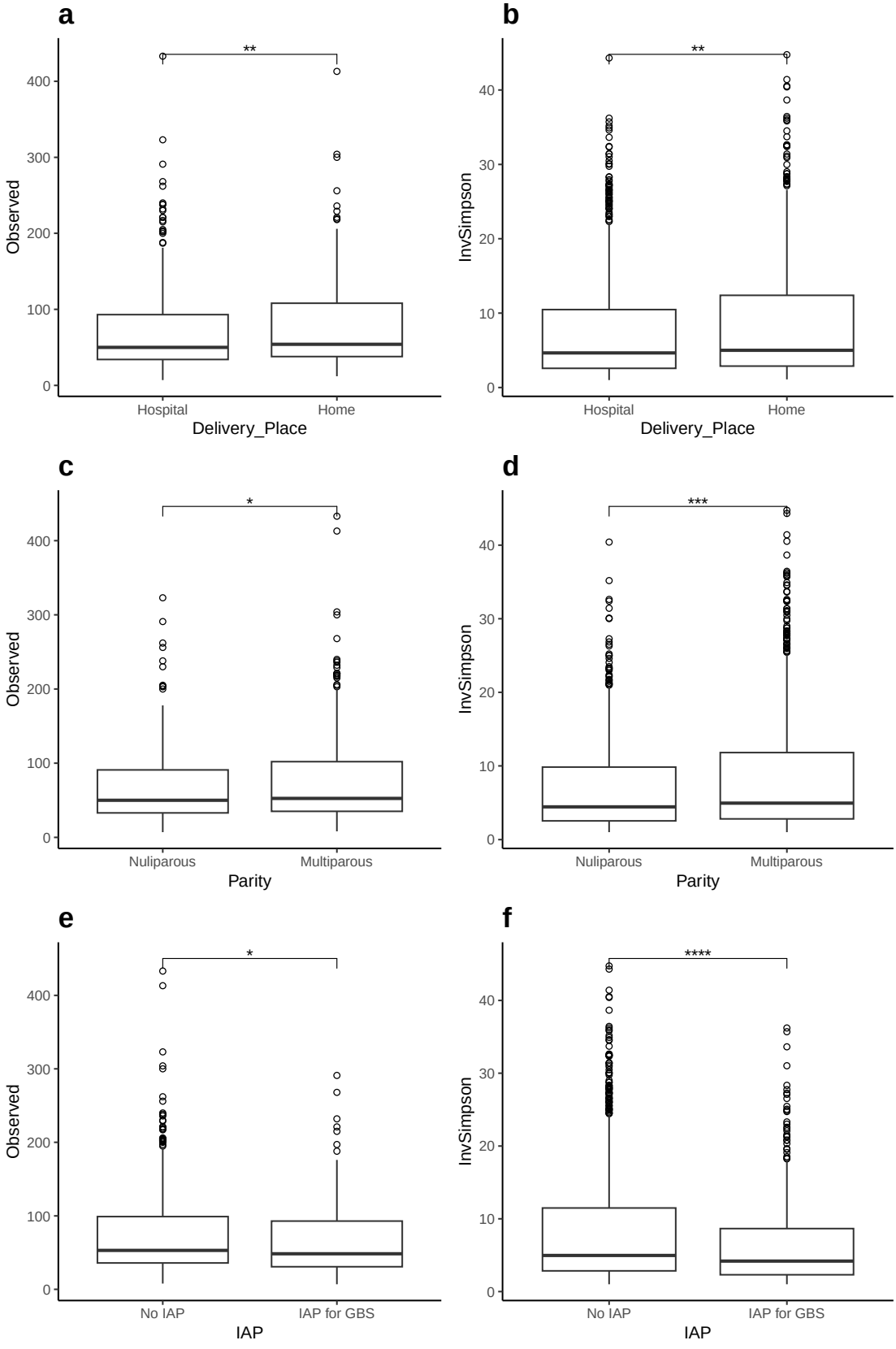
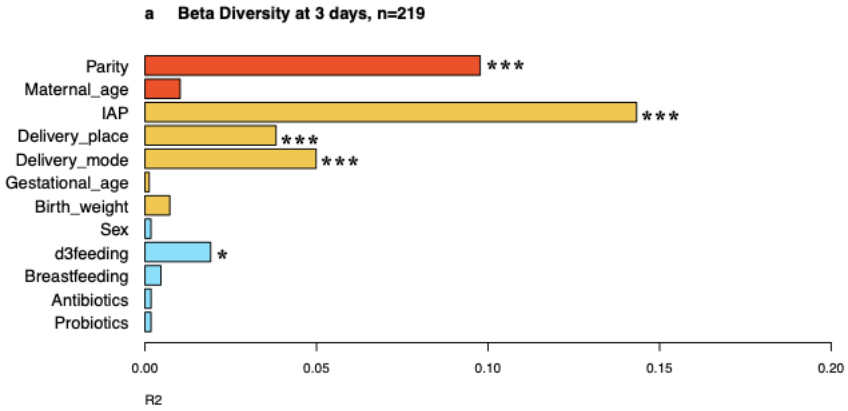
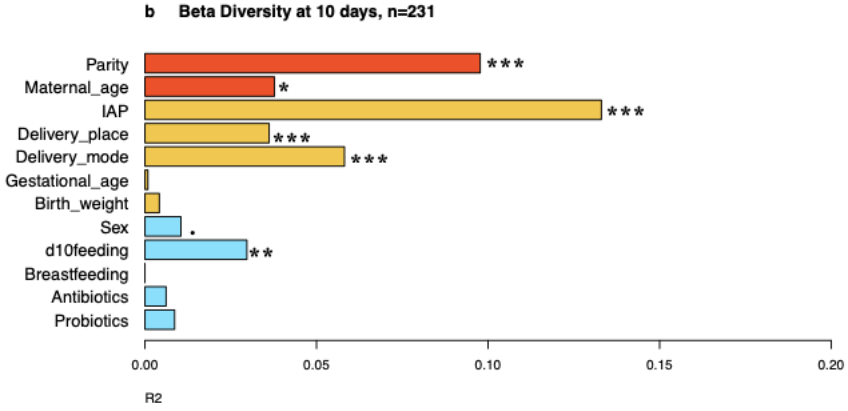
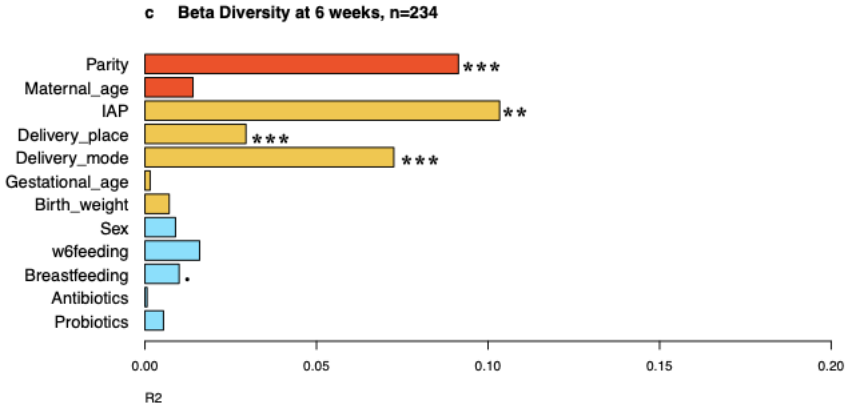
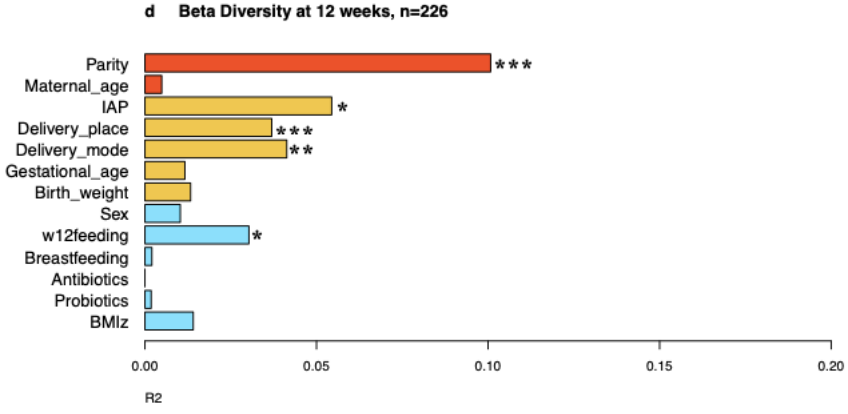

a
b
c
d
e
f
Observed
InvSimpson
Delivery_Place
Hospital
Home
Parity
Nuliparous
Multiparous
IAP
No IAP
IAP for GBS
**
*
***
****
0
100
200
300
400
10
20
30
40

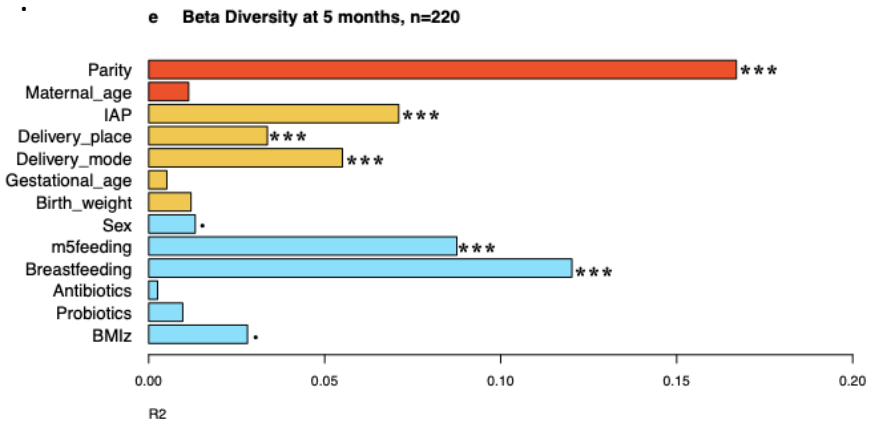
Figure 4: Delivery place, parity and IAP are associated with alpha diversity in the first three years of life. Boxplots of alpha diversity indices (Observed richness and Inverse Simpson) of all infants in the Baby & Mi cohort for the following exposures: (a-b) Delivery place, (c-d) Parity and (e-f) IAP for GBS. Each boxplot represents the average alpha diversity for all timepoints. All exposures are significantly associated with Observed richness and Inverse Simpson. The line inside each box represents the median value. Outliers are shown as dots and p-values are represented by asterisks. The significance code is: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

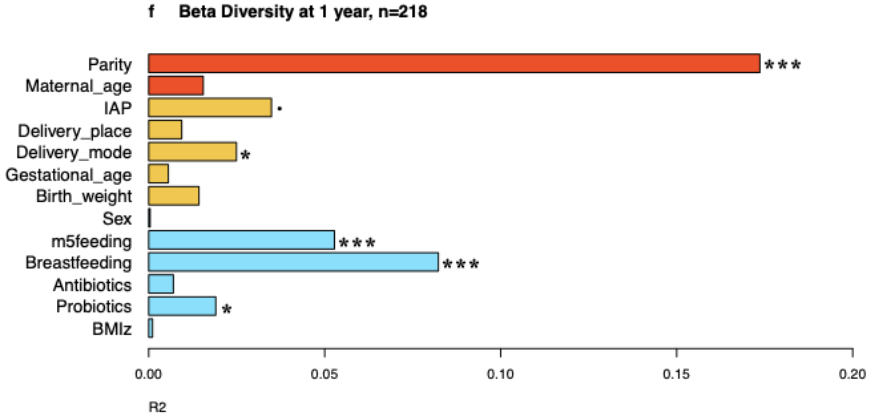

a Beta Diversity at 3 days, n=219
Parity ***
Maternal_age
IAP ***
Delivery_place ***
Delivery_mode ***
Gestational_age
Birth_weight
Sex
d3feeding *
Breastfeeding
Antibiotics
Probiotics
0.00 0.05 0.10 0.15 0.20
R2

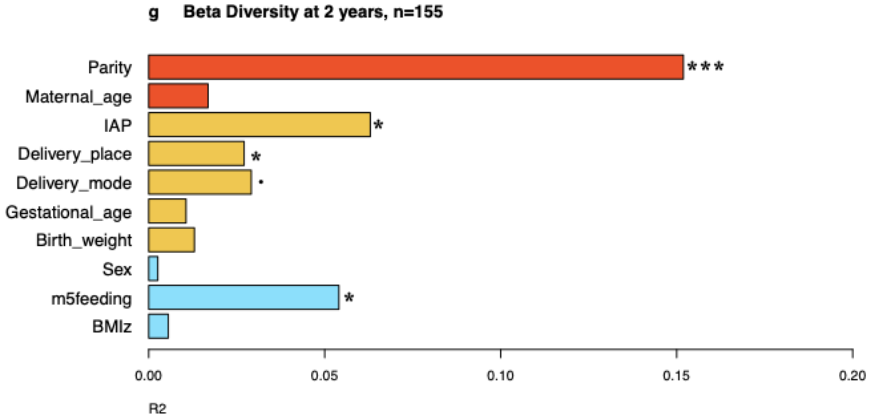

b Beta Diversity at 10 days, n=231
Parity ***
Maternal_age *
IAP ***
Delivery_place ***
Delivery_mode ***
Gestational_age
Birth_weight
Sex .
d10feeding **
Breastfeeding
Antibiotics
Probiotics
0.00 0.05 0.10 0.15 0.20
R2


c Beta Diversity at 6 weeks, n=234
Parity ***
Maternal_age
IAP **
Delivery_place ***
Delivery_mode ***
Gestational_age
Birth_weight
Sex
w6feeding
Breastfeeding .
Antibiotics
Probiotics
0.00 0.05 0.10 0.15 0.20
R2


d Beta Diversity at 12 weeks, n=226
Parity ***
Maternal_age
IAP *
Delivery_place ***
Delivery_mode **
Gestational_age
Birth_weight
Sex
w12feeding *
Breastfeeding
Antibiotics
Probiotics
BMIz
0.00 0.05 0.10 0.15 0.20
R2


e Beta Diversity at 5 months, n=220
Parity ***
Maternal_age
IAP ***
Delivery_place ***
Delivery_mode ***
Gestational_age
Birth_weight
Sex .
m5feeding ***
Breastfeeding ***
Antibiotics
Probiotics
BMIz .
0.00 0.05 0.10 0.15 0.20
R2


f Beta Diversity at 1 year, n=218
Parity ***
Maternal_age
IAP .
Delivery_place
Delivery_mode *
Gestational_age
Birth_weight
Sex
m5feeding ***
Breastfeeding ***
Antibiotics
Probiotics *
BMIz
0.00 0.05 0.10 0.15 0.20
R2


g Beta Diversity at 2 years, n=155
Parity ***
Maternal_age
IAP *
Delivery_place *
Delivery_mode .
Gestational_age
Birth_weight
Sex
m5feeding *
BMIz
0.00 0.05 0.10 0.15 0.20
R2

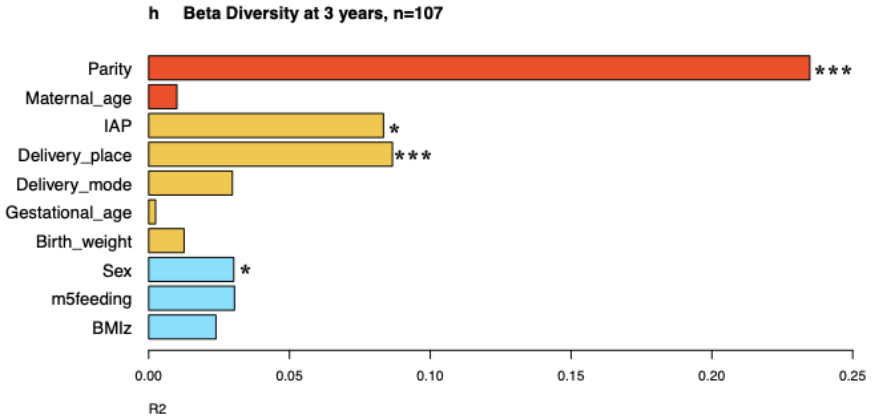

h Beta Diversity at 3 years, n=107
Parity ***
Maternal_age
IAP *
Delivery_place ***
Delivery_mode
Gestational_age
Birth_weight
Sex *
m5feeding
BMIz
0.00 0.05 0.10 0.15 0.20 0.25
R2

Figure 5: Delivery place, mode of delivery, parity, IAP and feeding practices are associated with beta diversity. The envfit model shows beta diversity associated with several covariates for the following timepoints: (a) 3 days, (b) 10 days, (c) 6 weeks, (d) 12 weeks, (e) 5 months, (f) 1 year, (g) 2 years and (h) 3 years. This model shows that delivery place, mode of delivery, parity, IAP, and breastfeeding are significantly associated with the variance in beta diversity of the microbiome ($p < 0.05$). Red, yellow, and blue represent maternal, perinatal, and infant variables, respectively. The R^2 values are plotted on the x-axis. p-values are represented by asterisks, and the significance code is: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

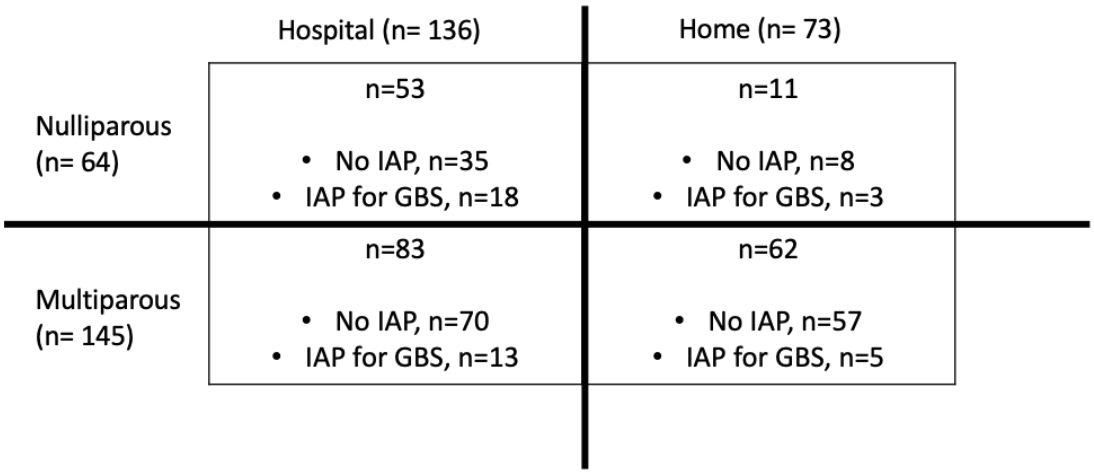


Figure 6: Stratification of infants based on delivery place, parity and IAP exposures. Diagram illustrating the stratification for all vaginally delivered infants (n= 209) according to three key variables: Delivery Place (Hospital vs Home Births), Parity (Nulliparous vs Multiparous), and IAP indication (no IAP vs IAP for GBS). Each box corresponds to a delivery place and parity subgroup with bullet points indicating the IAP status for each.

Table 2: Differentially abundant bacteria in Hospital vs. Home-born infants. Table showing differentially abundant taxa associated with Delivery Place for vaginally born infants of multiparous mothers not exposed to IAP (n=127).

TIMEPOINT	HOSPITAL (N=70)	HOME (N=57)
3d (n=120)	N/A	N/A
10d (n= 122)	*Haemophilus, f__Enterobacteriaceae, Escherichia-Shigella, Enterococcus, Enterobacter, Clostridium sensu stricto 1*	*Bacteroides*
6w (n=121)	*Clostridium sensu stricto 1*	*f__Lachnospiraceae*
12w (n=117)	N/A	N/A
5m (n=1)	N/A	*Bacteroides*
1y (n=121)	N/A	*Bacteroides*
2y (n=105)	*Blautia, [Ruminococcus] gnavus group*	N/A
3y (n=97)	N/A	N/A

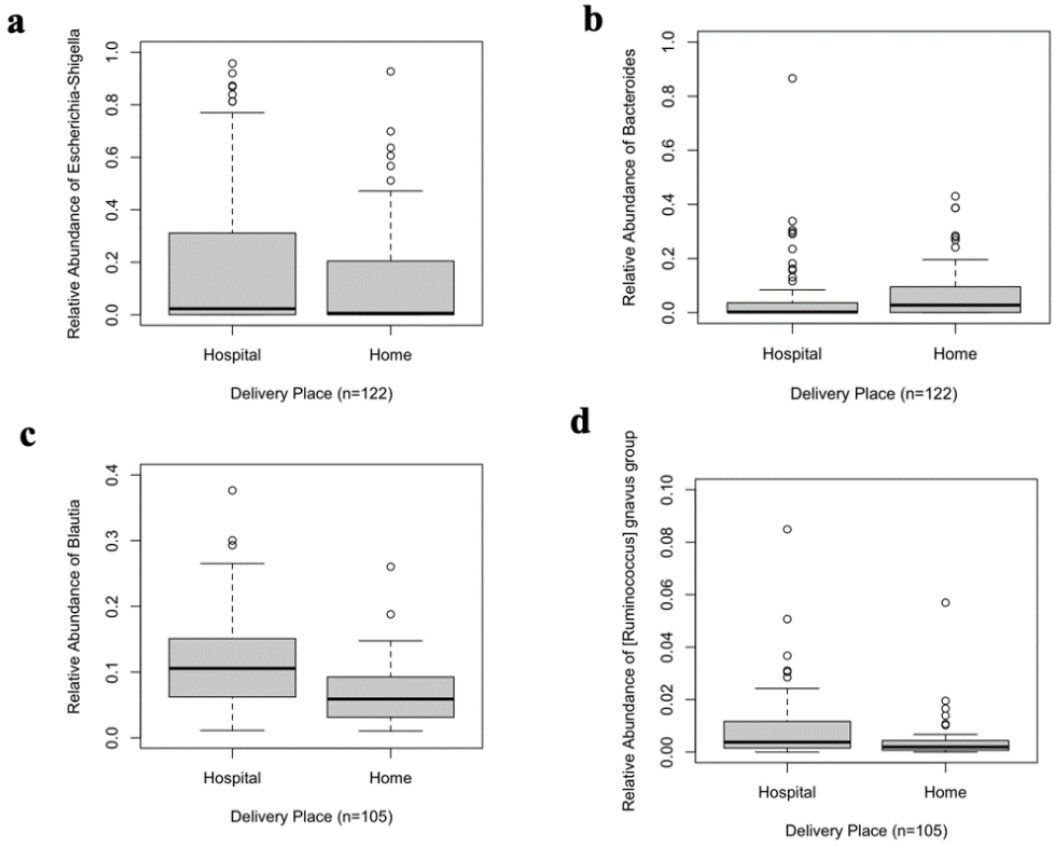


Figure 7: The relative abundance of taxa at 10 days and 2 years that are associated with delivery place. Relative abundance plots comparing taxa associated with the Hospital vs home birth exposure in a subgroup of infants of multiparous mothers not exposed to IAP. At 10 days: (a) *Escherichia-Shigella* and (b) *Bacteroides;* At 2 years: (c) *Blautia* and (d) *[Ruminococcus] gnavus group.* All taxa plotted are shown to be differentially abundant using DESeq2 with an adjusted p-value < 0.05. Taxa with a relative abundance of < 0.001 are excluded.

Table 3: Differentially abundant bacteria in infants of Nulliparous vs. Multiparous mothers. Table showing taxa associated with Parity for vaginally born infants that are hospital-delivered and not exposed to IAP (n=105). *Numbers in brackets next to taxa refer to the number of different ASVs for each genus.

TIMEPOINT	NULLIPAROUS (N=35)	MULTIPAROUS (N=70)
3d (n=97)	N/A	*Bifidobacterium* (3), *[Ruminococcus] gnavus group*
10d (n= 99)	*Parabacteroides*	*Streptococcus, Lacticaseibacillus, Escherichia-Shigella, Bifidobacterium* (2), *Bacteroides*
6w (n=100)	*f__Clostridiaceae*	*Staphylococcus, Gemella, Blautia, Bifidobacterium*
12w (n=98)	*Streptococcus, f__Clostridiaceae*	*Clostridium sensu stricto 1, Bifidobacterium, Bacteroides*
5m (n=91)	*Erysipelatoclostridium*	*Gemella, Bifidobacterium, Bacteroides*
1y (n=99)	N/A	*Suterella, Ruminococcus, Parasutterella, Parabacteroides, Lactococcus, Haemophilus, Erysipelotrichaceae UCG-003, Dorea, Butrycicoccus, Bifidobacterium, Allistipes*
2y (n=83)	N/A	*Suterella, Phascolarctobacterium, Bacteroides*
3y (n=74)	N/A	N/A

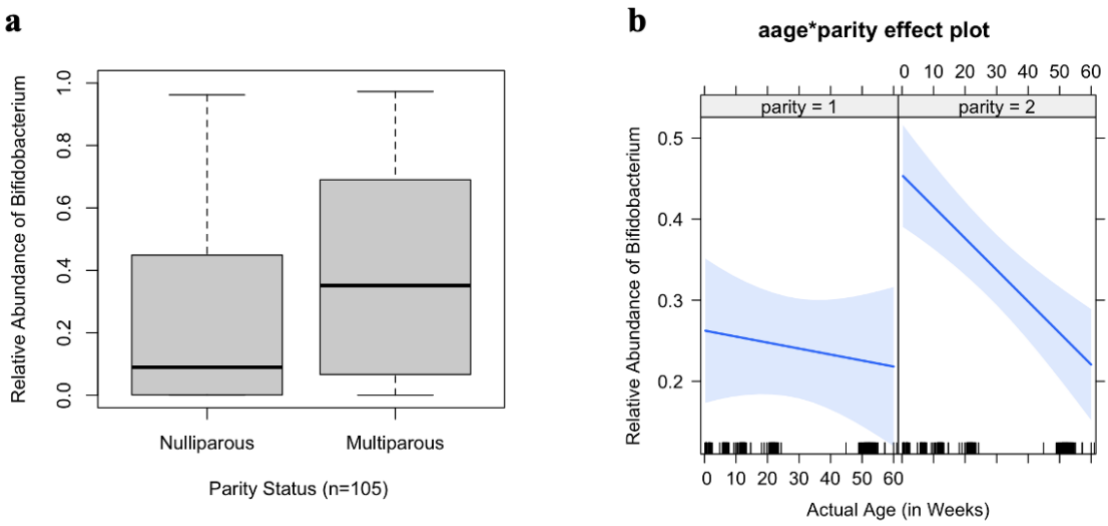


Figure 8: Infants of multiparous mothers have a higher abundance of *Bifidobacterium* over the first year of life. (a) Relative abundance plot of *Bifidobacterium* from 3 days to 1 year. (b) Age and parity linear effect plot showing the general trend in *Bifidobacterium* relative abundance over the first year. The difference in *Bifidobacterium* relative abundance is associated with parity ($p = 0.0008$) and a combined effect of parity and infant age ($p = 0.03$). The model is adjusted to account for interindividual variability. Parity 1 and 2 on the plot refer to infants of nulliparous and multiparous mothers, respectively.

Table 4: Differentially abundant bacteria in infants differ by IAP exposure. Table showing taxa associated with the IAP for GBS exposure for vaginally born infants of nulliparous mothers that were hospital-delivered (n=53). *Numbers in brackets next to taxa refer to the number of different ASVs for each genus.

TIMEPOINT	NO IAP (N=35)	IAP FOR GBS (N=18)
3d (n=48)	*Staphylococcus* (3), *Parabacteroides* (2), *Escherichia- Shigella, Enterococcus, Bifidobacterium, Bacteroides* (5)	*Streptococcus, Parabacteroides*
10d (n= 51)	*Staphylococcus, Pluralibacter, Parabacteroides* (2), *Bifidobacterium* (2), *Bacteroides* (5), *[Ruminococcus] gnavus group*	*Pectobacterium, f__Clostridiaceae*
6w (n=51)	*Streptococcus, Parabacteroides, f__Clostridiaceae, Escherichia-Shigella, Collinsella, Bifidobacterium* (3), *Bacteroides* (6)	N/A
12w (n=50)	*Parabacteroides, Escherichia-Shigella, Collinsella, Bifidobacterium* (2), *Bacteroides* (4)	*Clostridium sensu stricto 2, f__Clostridiaceae*
5m (n=47)	*Parabacteroides, f__Lachnospiraceae, Escherichia-Shigella, Collinsella, Bifidobacterium, Bacteroides* (7), *Akkermansia*	*Streptococcus, f__Clostridiaceae, Enterococcus*
1y (n=50)	*Ruminococcus, Roseburia, f_Lachnospiraceae, Bacteroides* (2)	*Lacticaseibacillus, Erysipelatoclostridium*
2y (n=43)	*Bacteroides*	N/A
3y (n=38)	*Streptococcus, Bacteroides* (3)	N/A

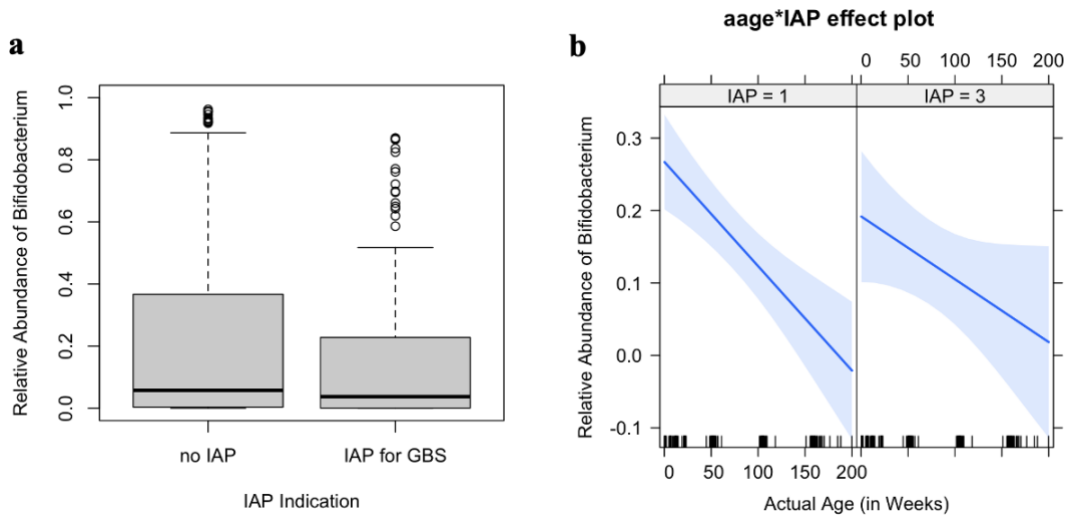


Figure 9: *Bifidobacterium* differs by IAP exposure over the first three years of life. (a) Relative abundance plot of *Bifidobacterium* from 3 days to 3 years. (b) Age and IAP linear effect plot showing the general trend in *Bifidobacterium* relative abundance over the first year. The difference in *Bifidobacterium* relative abundance is associated with infant age (p= 0.00007). The model is adjusted to account for interindividual variability. IAP 1 and 3 on the plot refer to infants with "no IAP" and those "exposed to IAP", respectively.

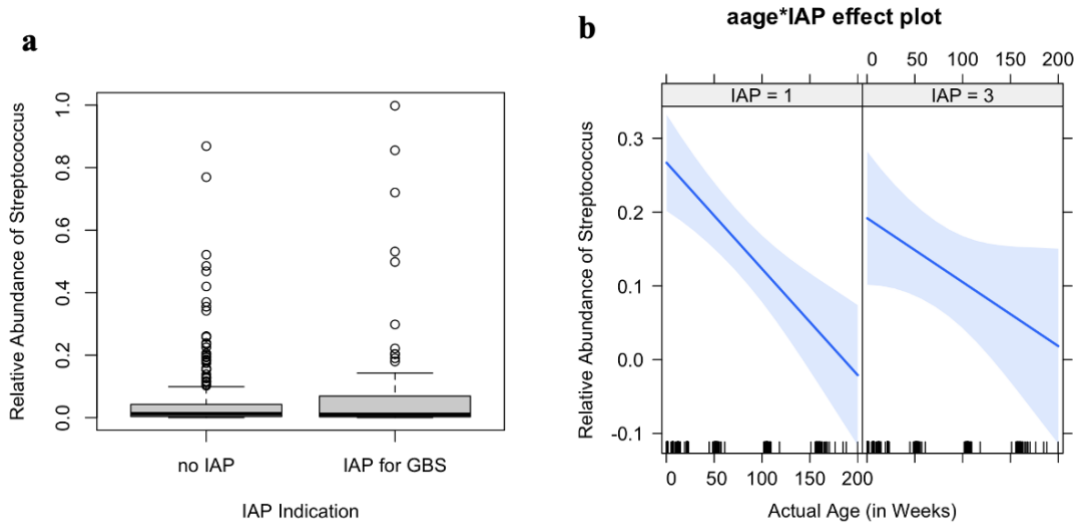


Figure 10: *Streptococcus* differs by IAP exposure over the first three years of life. (a) Relative abundance plot of *Streptococcus* from 3 days to 10 days. (b) Age and IAP linear effect plot showing the general trend in *Streptococcus* relative abundance over the first year. The difference in *Streptococcus* relative abundance is associated with infant age (p= 0.00007). The model is adjusted to account for interindividual variability. IAP 1 and 3 on the plot refer to infants with "no IAP" and those "exposed to IAP", respectively.

Table 5: Infants with Growth and Body Composition Data. Table showing the number of infants with BMIz, FMI, and LMI measures and those with missing data at all collection time points.

	Collection Timepoint				
	12 Weeks (n= 238)	**5 Months** (n= 238)	**1 Year** (n= 234)	**2 Years** (n= 210)	**3 Years** (n= 195)
BMIz (n)	233	234	230	196	176
Missing (n)	5	4	4	14	19
FMI (n)	202	163	N/A	N/A	58
Missing (n)	36	75	N/A	N/A	137
LMI (n)	202	163	N/A	N/A	58
Missing (n)	36	75	N/A	N/A	137

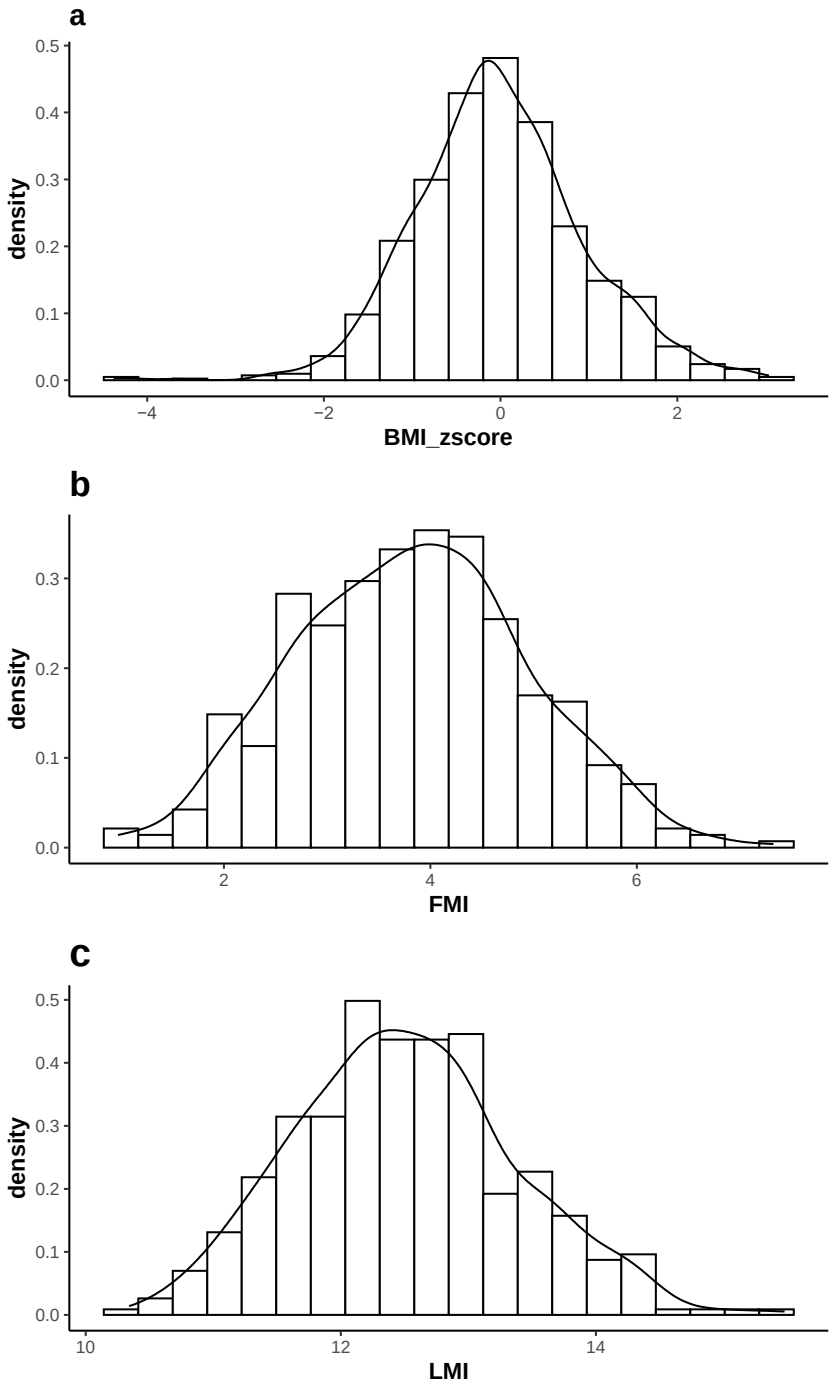


Figure 11: Infants' BMIz, LMI, and FMI follow a Gaussian distribution. Histogram density plots show the distribution of infants based on (a) BMIz, (b) FMI, and (c) LMI, with the density overlayed. Each plot represents infants' average BMIz, FMI and LMI at all timepoints from 3 days to 3 years.

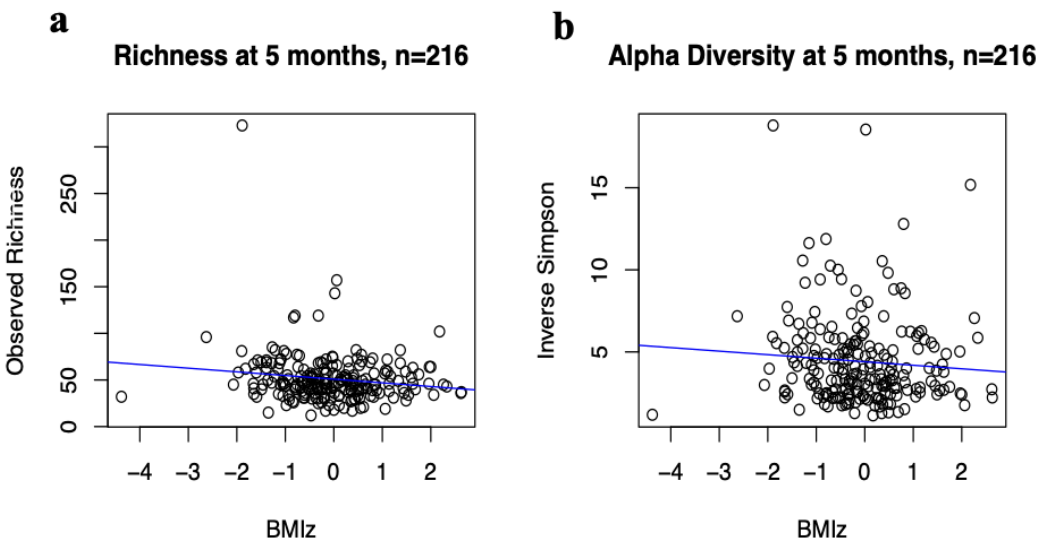


Figure 12: BMIz is associated with observed richness at five months. Boxplots at 5 months showing associations with BMIz with alpha diversity measures: (a) Observed richness and (b) Inverse Simpson. At 5 months, observed richness is inversely associated with BMIz ($p = 0.0327$). Infants with higher BMIz show lower gut microbiome alpha diversity. Trendlines are overlayed on the plots in blue.

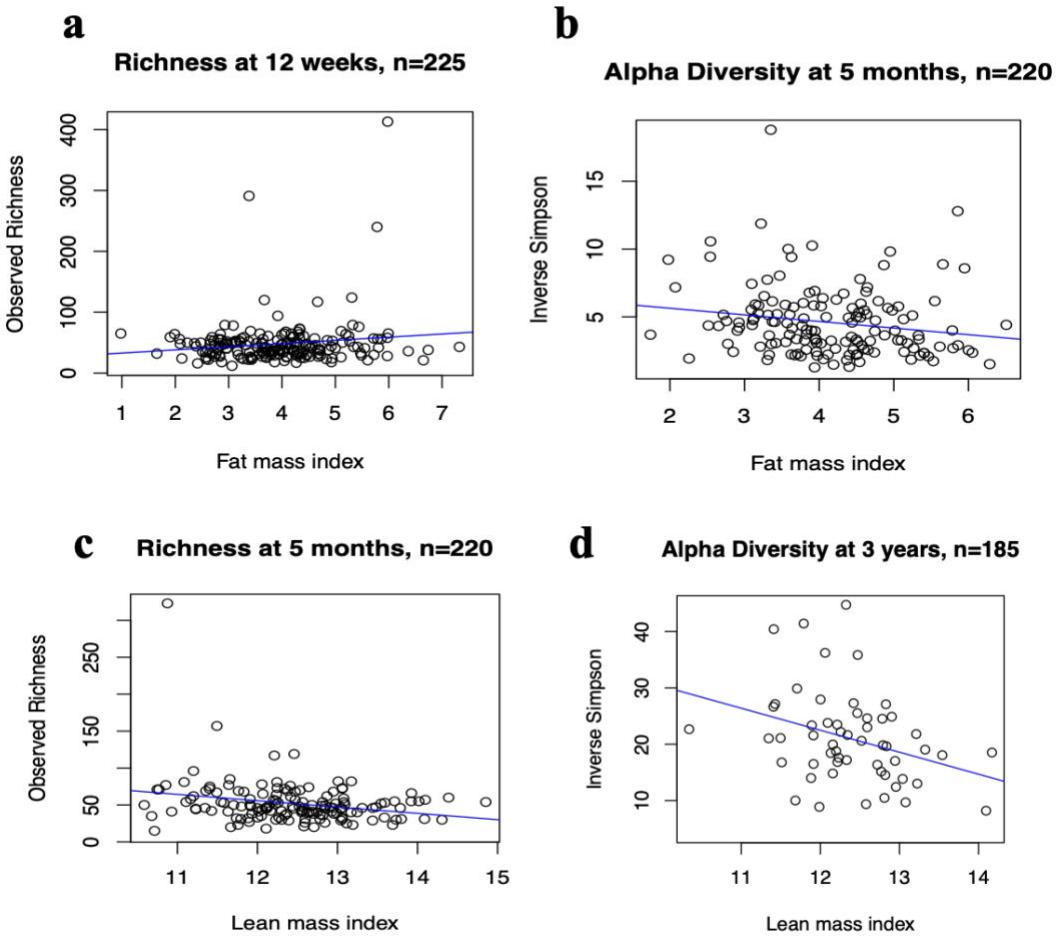
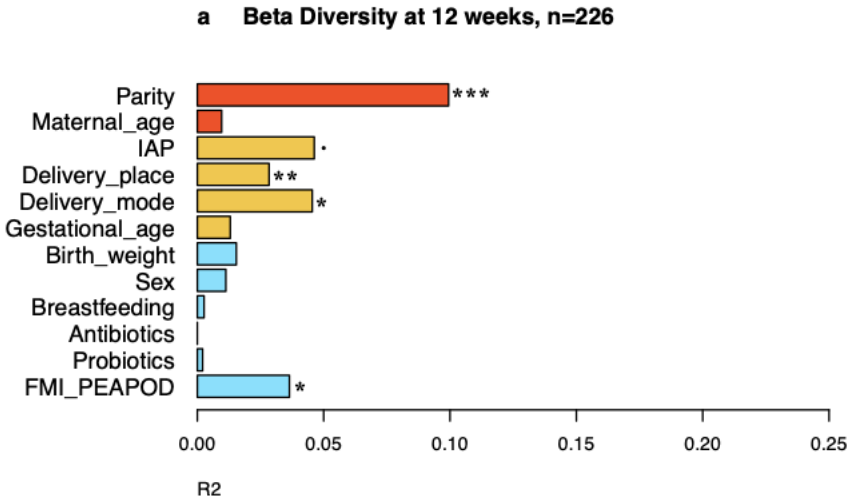
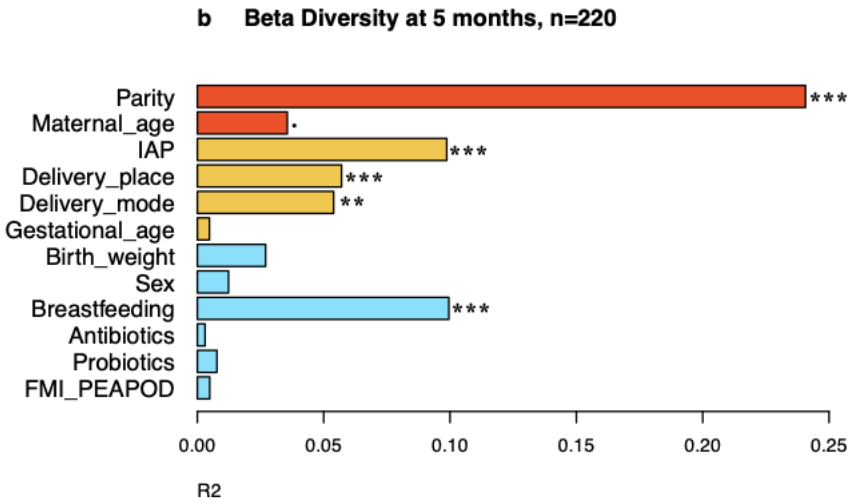


Figure 13: FMI and LMI are associated with alpha diversity at five months. Alpha diversity plots for infants with body composition measures (FMI and LMI) at 3 timepoints. Plots show the following: (a) FMI and Observed richness at 12 weeks, (b) FMI and Inverse Simpson at 5 months, (c) LMI and Observed richness at 5 months, and (d) LMI and Inverse Simpson at 3 years. FMI and LMI are inversely associated with alpha diversity at 5 months ($p < 0.05$). After accounting for maternal and perinatal covariates, FMI and LMI are not statistically associated with alpha diversity at 12 weeks and 3 years, respectively ($p > 0.05$). Trendlines are overlayed on the plots in blue.


a Beta Diversity at 12 weeks, n=226
Parity
Maternal_age
IAP
Delivery_place
Delivery_mode
Gestational_age
Birth_weight
Sex
Breastfeeding
Antibiotics
Probiotics
FMI_PEAPOD
***
.
**
*
*
0.00
0.05
0.10
0.15
0.20
0.25
R2


b Beta Diversity at 5 months, n=220
Parity
Maternal_age
IAP
Delivery_place
Delivery_mode
Gestational_age
Birth_weight
Sex
Breastfeeding
Antibiotics
Probiotics
FMI_PEAPOD
***
.
***
***
**
***
0.00
0.05
0.10
0.15
0.20
0.25
R2

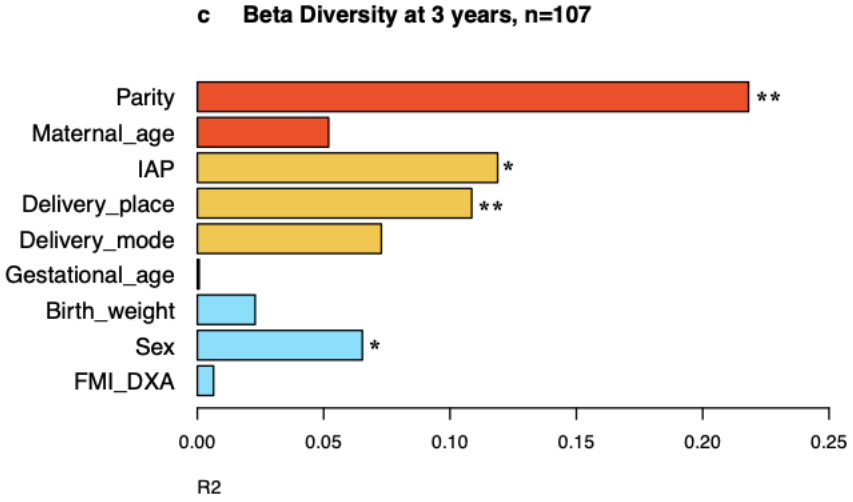

c Beta Diversity at 3 years, n=107
Parity
Maternal_age
IAP
Delivery_place
Delivery_mode
Gestational_age
Birth_weight
Sex
FMI_DXA
**
*
**
*
0.00
0.05
0.10
0.15
0.20
0.25
R2

Figure 14: FMI is associated with beta diversity at 12 weeks. The envfit model shows beta diversity associated with FMI and several covariates for the following time points: (a) 12 weeks, (b) 5 months, and (c) 3 years. This model shows that FMI, delivery place, mode of delivery, parity, IAP, and breastfeeding are significantly associated with the variance in beta diversity of the microbiome ($p < 0.05$). Red, yellow, and blue represent maternal, perinatal, and infant variables, respectively. The R^2 values are plotted on the x-axis. p-values are represented by asterisks, and the significance code is: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Figure 15: Microbiome beta diversity shows no clustering according to infant BMIz. PCoA plots showing Bray-Curtis dissimilarity distances overlayed with BMIz at the following timepoints: 12 weeks, 5 months, 1 year, 2 years and 3 years. A major shift in overall community structure is noted between 5 months and 3 years. No statistically significant clustering based on infant BMIz is observed ($p > 0.1$).

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