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‘Old Friends’ and learning to be friendly: How hygiene and social contact may affect the early-life gut microbiome and socioemotional development

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‘Old Friends’ and learning to be friendly: How hygiene and social contact may affect the early-life gut microbiome and socioemotional development

by

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1.0 – Abstract

The human gut microbiome is a fundamental factor in human health and wellbeing. The development of the microbiome during early infancy is a critical process that has long-term implications for brain development and social behaviour. However, modern life may be limiting exposure to important microbes due to decreased contact with a wide range of microorganisms. The unique circumstances of the COVID-19 pandemic further disrupted exposure, with increased hygiene practices and physical distancing policies limiting exposure to diverse microbes. The current study aimed to investigate the association between hygiene practices and social contact with features of the gut microbiome and metabolome in infants and their potential impact on socioemotional outcomes. Results showed that changes in social contact and hygiene practices associated with the diversity, composition and metabolomic environment of the gut microbiome in infants and there was evidence that changes in the gut microbiome associate with socioemotional development at 1 year of age. Additionally, the effect of hygiene practices on the microbiome was less than expected, while social contact proved to be a much more influential variable for the 3-month microbiome. Our findings suggest that the connections between early social interactions, the gut microbiome, and socioemotional development are complex, with social contact having a more significant impact than previously anticipated.

This thesis is original, unpublished, independent work by the author, D. Barth.

The experiments reported in Chapters 2-4 were covered by Ethics Certificate number 007, issued by the University of Calgary Conjoint Health Ethics Board for the project Pregnancy during the COVID-19 pandemic (REB20-0500) on 30th of March 2020.

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1. Introduction

1.1 – The Gut Microbiome in Health and Disease

The human gut microbiota is a diverse and complex community of trillions of microorganisms residing within the gastrointestinal tract. This community comprises a large variety of microorganisms, including bacteria, viruses, fungi, and archaea (Grice & Segre, 2011). These microorganisms have co-evolved with humans over millions of years, playing a fundamental role in human physiology, health, and overall wellbeing (Rook, Lowry, & Raison, 2013).

Colonization of the gut by microbes in early life is important to support healthy digestion and nutrient absorption, which is critical for growth and development (Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014). Recent research has also linked the gut microbiome to behavioural outcomes and normal brain development in various animal models, suggesting the gut-brain axis may be crucial in early cognitive and emotional development (Dinan & Cryan, 2017). While every individual has a distinct microbiome composition, most healthy people will share similar patterns of bacterial phylotype (groups of bacteria classified together by genetic or functional similarity) abundance and distribution in their intestines (Carabotti, Scirocco, Maselli, & Severi, 2015). Disruptions in the gut microbiota composition and function are associated with a range of health problems, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and obesity (Shreiner, Kao, & Young, 2015). Additionally, alterations in the gut microbiome during early life may increase the risk of developing asthma, allergies, and autoimmune disorders such as type 1 diabetes (Arrieta et al., 2014).

Given the crucial role the gut microbiome plays in human health and development, it is essential to understand how modern life and unique circumstances, such as the COVID-19 pandemic, impact its composition and function. This thesis aims to investigate the associations between hygiene practices, social contact, and the gut microbiome in infants, and their potential impact on socioemotional development.

1.2 – Driving Forces of the Developing Microbiome

The infant microbiome's development is a particularly dynamic process, which is more susceptible to change and modulation than the adult microbiome (Bokulich et al., 2016). This

susceptibility is due to several factors, including the lack of a stable microbiome during early life, the influence of maternal microbiota, and the rapid development of the infant's immune system (Round & Mazmanian, 2009).

The infant gut microbiome begins to develop from birth, with the infant's first exposure to microorganisms occurring during the delivery process, through contact with the mother and the birthing environment (Dominguez-Bello et al., 2010). The way a baby is born (whether vaginal or caesarean) changes the initial microbes the baby is exposed to, and differences in the composition of the early life microbiome can persist throughout the first few years of life (Jakobsson et al., 2014). Babies born vaginally get exposed to the mother's vaginal microbiota, while infants born by caesarean section may miss this exposure to the vaginal microbiome, instead receiving their foundational microbes through contact with the mother's skin, gut, and the hospital environment (Coelho et al., 2021; Dominguez-Bello et al., 2010).

Breast milk is another significant factor in the development of the infant microbiome. Breast milk contains bacteria like *Bifidobacterium* and *Lactobacillus* that colonize the infant's gut and contribute to a healthy microbiome (Fernández et al., 2013). With less direct exposure to *Bifidobacterium* and *Lactobacillus,* formula-fed infants have a gut microbiome with greater levels of bacteria like *Enterobacteriaceae* and *Bacteroides*. Commensal bacteria like *Bifidobacterium* and *Lactobacillus* maintain gut health and support immune function, while the inhibition of pathogenic bacteria helps to prevent infections and other adverse health outcomes (Fernández et al., 2013).

Gestational age at birth can also impact the trajectory of the infant microbiome (J. Penders et al., 2006). As preterm infants are primarily born by caesarean section, they may experience delayed colonization of their gut microbiome due to the lack of exposure to maternal vaginal and fecal microbiota during delivery (Arboleya et al., 2012). As a result, preterm infants may have a less diverse and less stable microbiome compared to full-term infants (Korpela et al., 2018).

Though antibiotics can be a lifesaving intervention, they are another factor that significantly affects the developing microbiome (Bokulich et al., 2016). Antibiotics can kill existing bacteria and inhibit the growth of bacteria in the body, often by indiscriminate mechanisms. This can lead to a decrease in microbial diversity and the loss of both pathogenic and commensal bacterial species in the gut due to the wide-ranging effect of the antibiotics

(Tanaka et al., 2009). Infants who receive antibiotics in the first year of life may have an increased risk of allergies, asthma, and obesity later in life (Bejaoui & Poulsen, 2020; Loewen, Monchka, Mahmud, t Jong, & Azad, 2018).

Environmental factors and social contacts, such as family members, also influence the early life microbiome following delivery. A study conducted by Valles-Colomer et al. on more than 9700 microbiome samples demonstrated that individuals who live together and share a household environment have a more similar gut microbiome composition than unrelated individuals (Valles-Colomer et al., 2023). Further, a study conducted by Laursen et al. of infants from the Danish SKOT cohort found that infants with older siblings had a more diverse microbiome compared to those who were the firstborn (Laursen et al., 2015). The researchers analyzed fecal samples from 114 infants and found that those with older siblings had higher diversity and richness in the microbiome at 18 months, with increasing numbers of older siblings also associated with increased diversity among *Bifidobacterium* and *Firmicutes* species. The researchers suggested that exposure to a wider range of microorganisms through contact with older siblings and altered parental hygiene behaviour may contribute to this increased diversity (Laursen et al., 2015). Pets, particularly dogs and cats, are also known to introduce a diverse range of microorganisms into the home environment (Kates et al., 2020). When infants are exposed to these microorganisms, either through direct contact with the pet or by sharing the same home environment, some of these microorganisms colonize the infant's gut and skin microbiome (Laursen et al., 2015; H. M. Tun et al., 2017). The colonization of the infant's microbiota by pet-associated microorganisms can have a positive impact on the development of the immune system, such as reducing the risk of allergies and other immune-related disorders, and can also increase microbial diversity and abundance of bacteria in the gut (H. M. Tun et al., 2017). While these environmental factors – primarily pets and parents – have been more extensively studied, the social context, such as interactions with family, community and outside social connections, remains less studied (Lane et al., 2019). This gap in research presents an opportunity to further explore how social contact shapes the early life microbiome.

1.3 – Mechanisms of The Gut-Brain Axis

As discussed, the gut microbiota plays a crucial role in regulating the immune system, metabolism, and brain function. In particular, the gut microbiota is increasingly linked to early

brain development and may influence behavioural outcomes as well as the development of mental health conditions like depression and anxiety (J. Kelly et al., 2015). These effects are at least partly mediated through the gut-brain axis, a two-way communication system between the central nervous system and the gut, allowing the microbiota to signal to the brain (Yang et al., 2016).

Microbes in the gut release a variety of bioactive compounds, including neurotransmitters, short-chain fatty acids and other chemical messengers that travel to the brain through the bloodstream and stimulate the vagus nerve (Dinan & Cryan, 2017). Through these mechanisms, they stimulate the release of hormones or influence the immune system to indirectly affect the brain. Different bacteria have different roles and create varying chemicals and nutrients, meaning the types of bacteria present in the microbiome can impact which messages are sent to the brain (Cryan & Dinan, 2012). Conversely, the brain can also influence the composition and activity of the microbiota through various mechanisms, including regulating gut motility and hormone secretion (Carabotti et al., 2015).

In early life, there is evidence that the gut-brain axis plays a crucial role in brain development, particularly in the development of neural circuits that are involved in social behaviour and emotion regulation (Dash, Syed, & Khan, 2022; Gacias et al., 2016; Hoban et al., 2016). Studies in animals have shown that disruptions in the gut microbiota during critical periods of development can lead to alterations in brain function and behaviour later in life (Gacias et al., 2016). Additionally, antibiotic-treated mice have been shown to have altered expression of transporters essential to the production of neurotransmitters involved in mood regulation such as serotonin (Fröhlich et al., 2016).

Studies in zebrafish have also demonstrated the critical role the microbiome plays in both shaping brain architecture and normal social behaviour. Rea et al. found that germ-free zebrafish embryos had significantly reduced neural gene expression in critical signalling pathways of the brain (Rea, Bell, Ball, & Van Raay, 2022). Bruckner et al. investigated how the microbiota affects the region of the zebrafish brain that controls social behaviour (Bruckner et al., 2022). They found that in germ-free zebrafish during the first week of development, the presence of microbiota influences microglia behaviour and gene expression. The microbiome was crucial for the later establishment of normal social behaviour in the study. Microglia are the brain's resident immune cells that modulate neuronal activity and remodel brain circuits to regulate brain

development and function (Li & Barres, 2018). They are responsive to microbial signals, allowing the gut microbiota to modulate microglial function and remodelling of neural circuits, thereby altering social behaviour in zebrafish (Bruckner et al., 2022; Rea et al., 2022).

The gut-brain axis is a critical pathway through which the gut microbiota can influence early brain development and socioemotional outcomes. However, further investigation is needed to fully understand the mechanisms underlying these effects within humans.

1.4 – Socioemotional Development and The Early Microbiome

In connection to the brain, recent research has highlighted the important role of the gut microbiome in brain development and mental health, suggesting that early gut colonization may have long-terms implications for cognitive and emotional development (Dash et al., 2022).

It is understood that the microbiome can affect both brain function and behaviour outcomes in animals. Studies in germ-free mice have shown that a lack of gut microbiota can alter social behaviour and stress responses compared to mice with a normal microbiome (Heijtz et al., 2011). Depression-associated bacteria have been found to induce characteristic physiological and behavioural features in rats after microbiome transplantation from human patients (Kelly et al., 2016). These animal findings suggest the microbiome is not merely a passive resident in the gut but an active participant in modulating neurological and behavioral outcomes.

In a study by Sgritta et al. on social behaviour in a mouse model of autism spectrum disorder (ASD), they found that ASD-like symptoms in maternal high-fat diet (MHFD) mice offspring were associated with alterations in the gut microbiome (Sgritta et al., 2019). The bacteria *Lactobacillus reuteri* was reduced in the microbiome of MHFD offspring mice, but administration of *L. reuteri* to the MHFD mice could rescue deficits in social behaviour. *L. reuteri* was found to promote social behaviour through increased production of oxytocin, a neuropeptide involved in social bonding and affiliative behaviour, and activation of the vagus nerve. The study's results highlighted the ability of specific bacterial strains to modulate sociability in mouse models and suggest that targeted modulation of the gut microbiome could offer avenues for treating social behavior deficits associated with neurodevelopmental disorders.

Timing may also be an important factor in the relationship between the gut microbiome and socioemotional development. A study by Desbonnet et al. colonized germ-free mice with a

conventional microbiome at different developmental time points (Desbonnet et al., 2015). They found that colonizing germ-free mice with a conventional microbiota during early development prevented alterations in brain architecture and social behaviour typical in germ-free mice, but this rescue effect was not observed when the microbiota colonization occurred in adulthood. The time sensitivity in this and other animal experiments indicates the microbiota may play a role in specific developmental periods to influence socioemotional development.

In humans, there is growing evidence to suggest that the gut microbiota may influence the development of mental health and behavioural outcomes. Many studies in adults have demonstrated links between alterations in the gut microbiota and increased risk of developing mental health conditions like anxiety and depression (Foster, Rinaman, & Cryan, 2017; Sherwin, Dinan, & Cryan, 2018). Fewer studies have explored the microbiome's role in early childhood temperament and socioemotional development. One such study by Fox et al., found that certain bacterial taxa at 1-3 weeks of age were predictive of temperament at 1 year of age (Fox. M, et. al., 2021). Beta diversity (i.e., the diversity of microbial communities between samples or individuals) and abundance of *Bifidobacterium* and *Lachnospiraceae* were positively associated with measures of extraversion at 12 months, while the abundance of *Klebsiella* was negatively associated. These results demonstrate relationships between the gut microbiome and early behavioural outcomes as markers of socioemotional development in children.

Despite progress made in understanding how the microbiome affects social behaviour and its development in humans, knowledge remains limited by the relatively small number of studies conducted in humans to date (Sherwin, Bordenstein, Quinn, Dinan, & Cryan, 2019). However, as the gut microbiome generates signals essential to normal social behaviour in animals and is associated with behavioural outcomes in humans, disturbances to microbial exposure likely also lead to altered socioemotional development in children (Agranyoni et al., 2021; Sarkar et al., 2020; Wu et al., 2021). Larger and more diverse human studies, particularly during crucial development windows, that can validate findings from animal models are essential to clarify the complex interactions between the microbiome and human social behavior.

1.5 – Old Friends & The Hygiene Hypothesis

The 'Old Friends' hypothesis proposes that modern life has reduced exposure to evolutionarily important microbes that promote healthy brain development (Rook et al., 2013).

This hypothesis emphasizes the importance of the symbiotic relationship between humans and microbes, as well as the role of the microbiome in shaping physiology.

Similar to the 'Old Friends' hypothesis, the hygiene hypothesis suggests that insufficient exposure to certain bacteria and viruses during early childhood may interfere with the development of many physiological systems and by extension, increase the risk of disrupting critical pathways for brain development (Finlay et al., 2021; Rook et al., 2013). As discussed, the microbiome aids in the development and maturation of the immune system in early life. The microbiome can influence the development of the central nervous system, through pathways such as the activation of immune cells (like microglia) and modulation of immune responses, production of signalling molecules or modulation of the blood-brain barrier (Cryan & Dinan, 2012; Li & Barres, 2018).

Changes in the gut microbiome caused by factors such as antibiotics, diet, and hygiene practices can impact the development of many physiological systems within the body and thereby additionally alter communication between the gut and the brain (Bresesti et al., 2022; Rook et al., 2013). The hygiene hypothesis suggests that inadequate exposure to certain types of bacteria and viruses during early childhood may adversely affect the gut microbiome's diversity and composition, ultimately affecting later immune function and brain health (Sonnenburg & Sonnenburg, 2019). Social contact (the different people with whom we share space in close physical proximity) and the child's physical environment are essential sources of microbes that shape the gut microbiome, so alteration to these exposures may have far-reaching developmental impacts (Ebrahimi, Khatami, & Mesdaghi, 2022).

1.6 – The Context of the COVID-19 Pandemic

The ubiquitous use of antibiotics and antimicrobial products has long prompted concerns that children may lack exposure to essential microbes (Romagnani, 2004). The COVID-19 pandemic has further altered routes of microbial exposure, raising concerns that changes to social and hygiene behaviour will have detrimental impacts on gut microbiome maturation and socioemotional development (J. Romano-Keeler, J. Zhang, & J. Sun, 2021).

The pandemic response involved extensive increases in hygiene practices as well as the enforcement of broad physical distancing policies that reduced social contact (J. Romano-Keeler et al., 2021). In Canada, the COVID-19 pandemic led to significant changes in daily life and

public health policies. The federal, provincial, and territorial governments implemented various measures to slow the spread of the virus, including promoting hygiene practices such as handwashing, increasing the use of hand sanitizers, and cleaning high-touch surfaces (Fahad, Saeha, Naylor, & Arthur, 2022). Additionally, physical distancing policies were enforced, which caused the limiting of gatherings and social contact outside individual households, the closing of non-essential businesses, and implementation of remote work and learning policies. These measures were implemented across Canada with varying stringency depending on provincial epidemiological situations (Fahad et al., 2022). These policies meant many families spent more time at home during the pandemic, increasing the frequency of various hygiene practices and likely reducing exposure to outside environmental microbes (Finger et al., 2021; Finlay et al., 2021). Together, increased hygiene practices, use of antimicrobials, and a decrease in social contact have the potential to prevent or limit exposure to the variety of microbes (the 'Old Friends') that children would normally be exposed to during early life (Ebrahimi et al., 2022; Finlay et al., 2021; J. Romano-Keeler et al., 2021).

Reduced in-person social activity and increased physical distancing may have translated to decreased opportunities for infants to interact with other people and their surrounding environment. These changes could have several potential impacts on the infant microbiome. Socialization, referring to the interaction of infants with other individuals in close physical proximity, exposes infants to a wide range of environmental microbes, both from other people and from the surrounding environment (Finlay et al., 2021; Pasquaretta, Gómez-Moracho, Heeb, & Lihoreau, 2018). This exposure is important for developing a healthy gut microbiome, as it establishes a diverse microbial community in the infant's gut (Ebrahimi et al., 2022). Infants also acquire many of their gut microbes from their mother during birth and breastfeeding. However, socialization with other people also impacts maternal microbial transfer, as mothers may be exposed to new microbes from other people and the environment (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015; J. Romano-Keeler et al., 2021).

Further, the COVID-19 pandemic has had a significant effect on maternal mental health and wellbeing, which could indirectly influence the infant microbiome (Jašarević et al., 2018; Tomfohr-Madsen, Racine, Giesbrecht, Lebel, & Madigan, 2021). Maternal stress and mental health disorders, such as anxiety and depression, have been associated with alterations in the maternal gut microbiome, which can subsequently impact the infant gut microbiome through

transmission during birth and breastfeeding (Galley et al., 2023). Changes in social behaviour and maternal stress, combined with the increased use of antimicrobials, have the potential to alter the early life microbiome and socioemotional development of Canadian infants (Finlay et al., 2021). The true impact of the pandemic and varied levels of hygiene and social restrictions on shaping the early life microbiome and the implications for socioemotional development has so far remained unstudied (Ebrahimi et al., 2022).

1.7 – Aims & Hypotheses

The gut microbiome is essential to early brain development and social behaviour. The 'Old Friends' hypothesis suggests that modern life has reduced exposure to evolutionarily important microbes that promote healthy brain development, and disturbances to microbial exposure may lead to altered socioemotional development in children. The use of antibiotics and antimicrobial products has also raised concerns about children missing exposure to essential microbes. The COVID-19 pandemic has further altered routes of microbial exposure, with concerns that changes to social and hygiene behaviour will have detrimental impacts on the maturation of the gut microbiome and socioemotional development. We hypothesize that as hygiene habits increase and social contacts outside the home decrease, there was a reduced variety in children's gut bacterial taxa, leading to missing microbes that are associated with socioemotional outcomes.

Research Objective:

I aim to investigate the potential impact of increased hygiene practices and reduced social contact (physical proximity to others) during the COVID-19 pandemic on the gut microbiome and socioemotional development in infants.

Overarching Hypothesis:

Increased hygiene habits and reduced social contact due to the COVID-19 pandemic have the potential to prevent or limit exposure to essential microbes, resulting in a less diverse gut microbiome and potential alterations in socioemotional development in children.

Aims:

First aim:

1. Investigate if increased hygiene habits and reduced social contact are associated with a lower alpha diversity (i.e., mean species diversity) of the gut microbiome, as well as other microbiome measures (i.e., beta diversity, specific taxa, metabolomic pathways). We predict that, as hygiene habits increase and social contacts decrease, there will be a reduced variety in children's gut bacterial taxa.

Second aim:

2. Seek to identify microbes that are present in the stool of infants exposed to less intensive hygiene and/or greater external social contact but not present in the stool of infants exposed to more intensive hygiene and/or fewer social contacts (i.e., 'missing microbes').

Third aim:

3. Determine whether lower alpha diversity and other microbiome features such as specific taxa or functional pathways predict socioemotional outcomes at one year of age.

Fourth aim:

4. Investigate if there is also an indirect effect of social contact and hygiene habits on socioemotional development, mediated by aspects of the gut microbiome such as alpha diversity or missing microbes. We predict that social contact at 3 months of age will have a positive relationship with ASQ: SE-2 scores at 1 year of age.

Fifth aim:

5. Investigate the functional component of the gut microbiome through metabolomics, as relating to the aims detailed above.

2. – Methods

2.1 – Participant Recruitment

All data used in the project is from a sub-study (n=873) of infants in the *Pregnancy During the Pandemic* (PdP) study which investigates the effects of the COVID-19 pandemic on mental health among pregnant women (Giesbrecht et al., 2021). Participants were eligible for the larger PdP study if they were pregnant, ≤35 weeks gestation at study enrollment, ≥17 years old, living in Canada, and able to read and write in English or French (Giesbrecht et al., 2021). Participants were recruited via ads on Facebook and Instagram in both French and English. Study enrollment, consent, and administration of questionnaires were conducted through Research Electronic Data Capture REDCap (Harris et al., 2009). Participants did not receive compensation for participating in the intake survey. This study received ethics approval (REB20-0500) from the University of Calgary Conjoint Health Research Ethics Board on March 26, 2020. All participants signed the electronic informed consent form prior to providing any data.

A subsample (N = 873) of pregnant individuals was recruited from the PdP study to collect their infants' biosamples. Exclusion criteria prior to sampling included use of antibiotics or vaccinations received in the last two weeks, as well as at the time of sampling the infant may not have any symptoms of flu or COVID-19. Samples were collected between October 21st, 2020 and April 25th, 2022.

2.2 – Sample Demographics:

Table 1. Sample Demographic Characteristics.

* Though some babies were from pregnancies of multiples, stool samples were only collected from 1 child per pregnancy.

** Mothers could identify more than 1 feeding status when responding.

	n		%
Infants (n = 873) *			
Sex	436 Female / 437 Male		49.9 % / 50.1 %
Preterm Infants (GA at birth < 37 Weeks)	32 Preterm / 841 Full-Term		3.7 % / 96.3%
Delivery Method	621 Vaginally / 252 Caesarean Section		71 % / 29 %
Antibiotic Exposure After Birth	78 Taken Antibiotics After Birth / 762 No Antibiotics / 33 Missing		8.9 % / 87.3 % / 3.8 %
Presence of 1 or more siblings	480 Single Children / 391 With Sibling / 2 Missing Data		55 % / 44.8% / 0.2%
Breastfeeding Status at 3 Months (Week Prior to Stool Sampling) **	Breast milk from breast	714	81.6 %
	Breast milk from bottle	262	29.9 %
	Formula	219	25.0 %
	Other	159	18.2 %
	Missing	30	3.4%
Total Household Income (Before Taxes)	Less than $20, 000	9	1.0%
	$20,000- $39,999	19	2.2%
	$40,000-$69,999	68	7.8%
	$70,000-$99,999	142	16.3%
	$100,000 -$124,999	164	18.8%
	$125,000- $149,999	138	15.8%
	$150,000 - $174,999	136	15.6%
	$175,000- $199,999	80	9.2%
	$200,000+	116	13.3%

	Missing	1	0.1%
Education	Less than high school diploma	1	0.1%
	Completed high school	32	3.7%
	Completed trade, technical, vocational school or business/community college	109	12.5%
	Bachelor's degree	368	42.2%
	Master's degree	234	26.8%
	Doctorate (PhD)	55	6.3%
	Professional (MD, JD, DDS, ETC)	71	8.1%
	Missing	3	0.3%
Maternal Ethnicity	Caucasian	736	84.3 %
	First Nations/Inuit	7	.8 %
	Metis	21	2.4 %
	Black	3	.3 %
	West Asian	8	.9 %
	South Asian	16	1.8 %
	Southeast Asian (incl. Filipino)	13	1.5 %
	East Asian (incl. Chinese, Korean)	19	2.2 %
	Hispanic/LatinX	17	1.9 %
	Biracial	33	3.8 %
	Range		Mean ± SD
Gestational Age at Birth (months)	32 - 43		39.47 ± 1.44

Birth Weight (grams)	1500 - 5160	3444.191
Infant Age at Stool Sampling (in Months)	.39 – 5.85	3.07 ± 0.31
Maternal Age at Intake (years)	18.75 – 45.42	33.33 ± 3.94

2.3 – Data Collection for Exposure Variables (Hygiene Practices and Social Contacts)

Demographic data was collected through online questionnaire at time of recruitment using REDCap electronic data capture tools hosted at the University of Alberta (Harris et al., 2009). To measure the exposure variables of hygiene habits and extent of social contact, participants completed questionnaires at 3 months of age about the variety and extent of the infant's social contacts as well as handwashing and cleaning practices. The questions that were used to create an individual score for each participant are outlined in Table 2 and 3 below with their scoring criteria.

Table 2. Survey Questions Relevant to Hygiene Practices

Questionnaire Items	**Question Response Options**
How many times per day do you wash your hands?	*0, 1-3, 4-6, 7-9, ≥ 10*
What do you usually use for handwashing? Check all that apply.	*Water without soap, Regular soap, Antibacterial soap, Antibacterial gel, Other*
How often do you wash your hands before you touch your baby?	*Always, Most of the time, Some of the time, Rarely, Never*
Do you clean your baby's pacifier when it falls on the floor/ground?	*Always, Most of the time, Some of the time, Rarely, Never*
Of these times, how often do you sterilize your baby's pacifier after it falls on the floor/ground?	*Always, Most of the time, Some of the time, Rarely, Never*
How often do you wash your baby's toys?	*More than 4 times per day, 2-3 times per day, Once per day, 4-6 times per week, 2-3 times per week, Once per week or less*
Of these times, how often do you sterilize your baby's toys?	*More than 4 times per day, 2-3 times per day, Once per day, 4-6 times per week, 2-3 times per week, Once per week or less*
How often are most of the surfaces of your home vacuumed/cleaned?	*More than once per day, Once per day, More than once per week, Once per week, Every other week, Less than every other week*
Are there any pets in your household? If yes, please specify: type of animal, how many, long hair	*Written answer, Y/N*
Do you live on a farm?	*Y/N*
Do you keep animals on your farm?	*Y/N*

Table 3. Survey Questions Relevant to Level of Social Contact

Questionnaire Items	**Question Response Options**
How often have visitors (non-household members) come to visit your house in the last 2 weeks?	*More than once per day, Once per day, More than once per week, Once per week, Once in the last 2 weeks, None in the last 2 weeks*
How often did you take your baby to public places (e.g. parks, restaurants, shopping malls) in the last 2 weeks?	*More than once per day, Once per day, More than once per week, Once per week, Once in the last 2 weeks, None in the last 2 weeks*
How often did you take your baby to visit other households in the last 2 weeks?	*More than once per day, Once per day, More than once per week, Once per week, Once in the last 2 weeks, None in the last 2 weeks*
Over the past two weeks, on average, who has touched/held your baby? Please indicate how often and whether they are household members. Options are: Yourself, Baby's Father/Your Spouse, Sibling(s), Grandparent, Aunt/Uncle, Nanny, Friend, Other	*Check boxes: Household member?* *Frequency: Daily, weekly, less than weekly*

2.4 – Score Creation:

Following extensive data cleaning and validation, a network analysis was conducted to explore the interrelationships among the questionnaire variables (see Figure 1). Based on the network analysis results and theoretical considerations, composite scores for social contact and hygiene practices were created:

Hygiene Practices Score: The hygiene-related variables showed some associations, though not as distinctly as the social contact variables. Guided by literature and theory, hygiene practices were characterized into surface-related and non-social variables. An additive index score was then created to reflect the cumulative effect of these practices. This score included items on handwashing frequency, handwashing product type, handwashing before touching the baby, pacifier cleaning and sterilization frequency, toy cleaning and sterilization frequency, and surface cleaning frequency.

Social Contact Score: Variables related to social contact clustered together clearly in the network analysis. To capture this underlying construct, a mean score for each participant was created. This score was derived from questions about the frequency of visitor interactions, public place visits, other household visits, and who had contact with the baby (including household members and non-household members).

Individual Variables: The variables for living on a farm and having a furry pet did not exhibit strong associations with other variables. Therefore, they were treated as individual scores to capture their unique contributions.

Considerations for Missing Data: Approximately 200 participants did not report using pacifiers or toys. A t-test indicated that these participants had lower hygiene scores. Despite this, we retained these items in the score calculation due to their significance as potential routes of environmental microbe exposure.

Score Correlations: Correlation analyses were conducted between the social contact score, hygiene practices score, and various relevant covariates and demographic factors, to explore how these variables individually related to the two scores. A very weak positive correlation was found between household income and hygiene scores ($\rho = 0.1159$, $p = 0.0006$), as well as between education level and hygiene scores ($\rho = 0.0904$, $p = 0.008$). A very weak negative correlation was observed between hygiene score and the presence of siblings ($\rho = -0.1020$, $p = 0.0027$). Similarly, a very weak negative correlation was found between social

contact score and education level (ρ = -0.0923, p = 0.0068), and between social contact score and method of delivery (ρ = -0.0809, p = 0.0174). On the other hand, there was a strong positive correlation between social contact score and the presence of siblings (ρ = 0.6723, p < 2.2e-16), which is expected as siblings are one of the variables included in the social contact score. Additionally, a very weak positive correlation was found between social contact score and maternal age (ρ = 0.1085, p = 0.0014). All other correlations were not statistically significant.

Contribution of Individual Variables to Overall Score: To investigate which variables were the primary contributors for each score, we performed a Principal Component Analysis, with all hygiene-related variables scaled to have a mean of zero and a standard deviation of one. We computed the correlation matrix using the cor function to assess variable relationships. PCA was conducted on the matrix (to manage multicollinearity by capturing shared variance among variables) using the prcomp function, and the principal components were summarized to determine variance explained (Ringnér, 2008). Loadings of the variables on the first two principal components were examined to understand their contributions. Cos2 values indicate how well each variable is represented in the PCA space defined by the first two dimensions, with higher values indicating better representation (Abdi & Williams, 2010).

For the hygiene practices score, frequency of washing hands had the highest quality of representation (Cos2 = 0.15). Washing hands before touching the baby was the next most significant behavior (Cos2 = 0.11). Other factors such as sharing the same bed (Cos2 = 0.07), the type of handwashing product used (Cos2 = 0.06), using a pacifier (Cos2 = 0.04), frequency of surface cleaning (Cos2 = 0.04), and cleaning toys (Cos2 = 0.02) showed lower representation.

The analysis of social variables revealed that having siblings had the highest quality of representation (Cos2 = 0.13). The frequency of visits to public places was the next most significant variable (Cos2 = 0.10). Other notable contributors included the frequency of visitors to the household (Cos2 = 0.09) and the frequency of visiting other households (Cos2 = 0.09). Variables such as the number of household members (Cos2 = 0.06) and the frequency of interactions with household members (Cos2 = 0.03) showed lower representation.

Score distributions are shown in Figure 2 and 3 below.

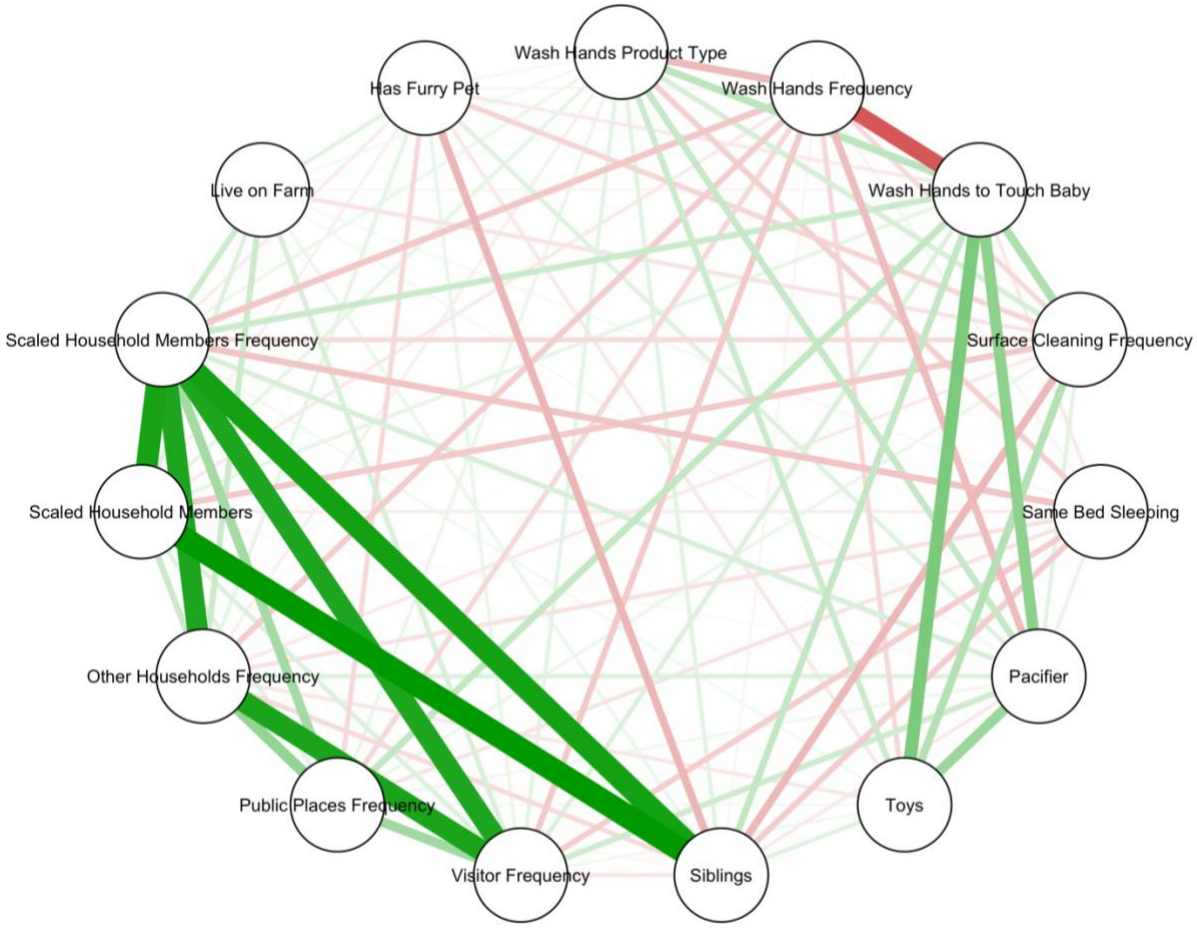

Figure 1. Network Analysis of Social and Hygiene Variables. *Green lines indicate a positive association between variables, with the line thickness indicating the strength of the relationship. Red lines indicate a negative association, with line width indicating strength of negative association.*

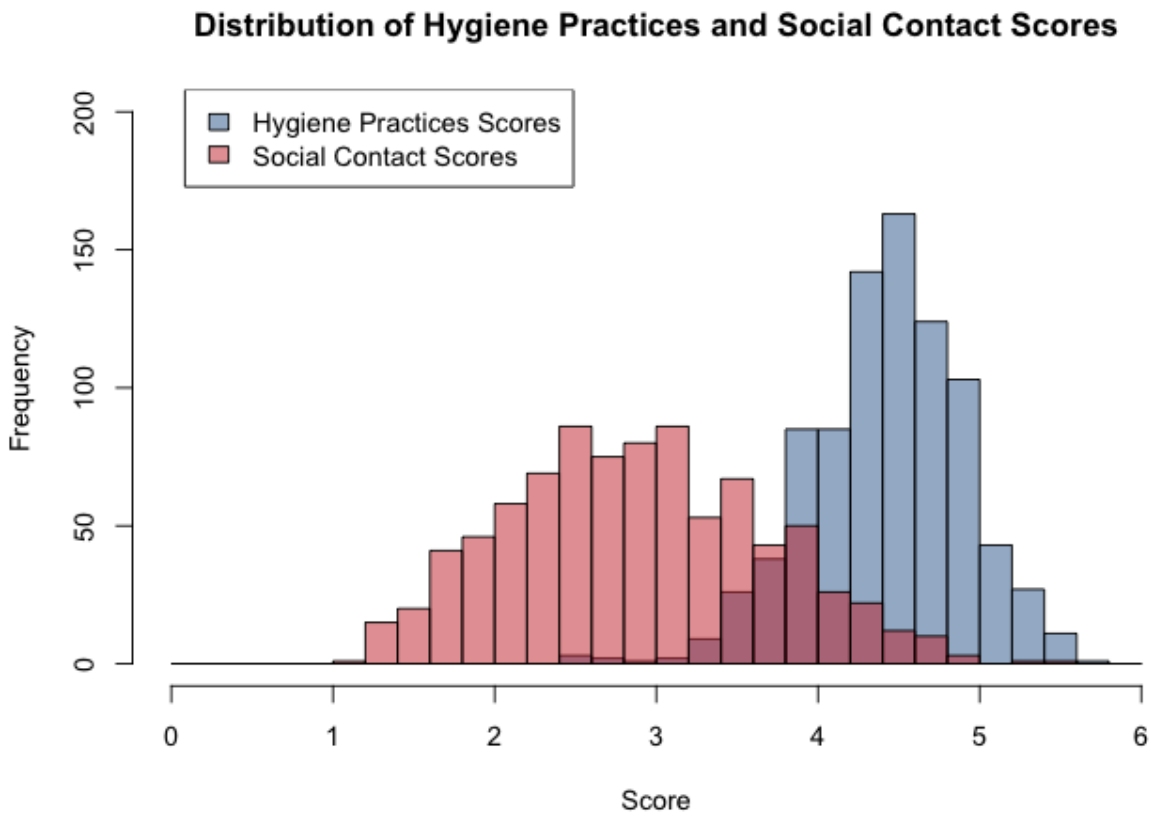

Figure 2. Histogram Diagrams of Hygiene Practices and Social Contact Score Distributions. *Hygiene Practices score distributions are pictured in blue, while Social Contact score distributions are shown in red (n = 873). Overlap is shadowed. Each bin represents a 0.2 increment in scores.*

2.5 – Primary Outcome: Ages & Stages Questionnaire – Socioemotional Development

To measure socioemotional outcomes, the Ages & Stages Questionnaire – Socioemotional Development, Second Edition (ASQ: SE-2), (Squires, Bricker, & Twombly, 2002) was assessed at one year of age, with data collected using REDCap . The ASQ: SE was chosen in this study as it is a widely recognized and reliable screening tool, specifically designed to assess socioemotional development in children and has strong associations with long term outcomes (Squires, Bricker, & Twombly, 2002). The ASQ: SE is organized into age-specific questionnaires, with each questionnaire having a different number of items, depending on the age of the child. The total score for each questionnaire is derived by summing up the individual item scores. At 1 year of age (12 months), the ASQ: SE consists of 27 items. Each behavioural item is scored on a scale ranging from "Often or always" (0 points), "Sometimes" (5 points), or "Rarely or never" (10 points). Higher total scores indicate greater social-emotional concerns or potential developmental problems (Squires, Bricker, & Twombly, 2002).

Both continuous and cutoff approaches were employed for analyzing the ASQ: SE scores to capture both nuanced variability and clinical relevance. The continuous (referred to as 'raw') scores allowed for a more granular assessment of socioemotional development by treating the total score as a spectrum, which can capture subtle differences. The cutoff approach is used to categorize scores into distinct risk groups (e.g., below cutoff, monitor, and refer), providing a clinical interpretation of whether a child might be at risk for socioemotional difficulties (Squires, Bricker, Twombly, et al., 2002).

2.6 – Stool Sample Collection

Infant stool samples were collected at home, at 3 months of age. Samples were collected using a microbiome (DNAGenotek OM-200) and metabolome (DNAGenotek, ME-200) stool collection kit and shipped back to the lab at the University of Calgary for analysis. The OM-200 kit stabilizes the stool samples for at least 60 days, while the ME-200 kit stabilizes stool samples for at least 30 days.

Gut Microbiome Metagenomic Sequencing and Processing:

Samples were analyzed using shotgun metagenomics sequencing at the International Microbiome Center (IMC) in Calgary. 500 ul of fecal sample was aliquoted from the omnigut

tube into PowerBead tube (Qiagen 19301). DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen, CA) following manufacturer's protocol. The concentration of extracted DNA was estimated with Quant- iTTM PicoGreenTM dsDNA Assay Kit, (Life Technologies, USA). Individual libraries were prepared with 100 ng of DNA as input and barcoded with unique dual indexes using 2S® TURBO DNA library and Normalase UDI ® kits from Swift Biosciences (MD, USA). Pooled library was sequenced on Illumina Novaseq 6000 S1-300 (150 base pairs paired end). For quality control of shotgun metagenomics, negative control and a mock community was sequenced with the samples.

Fastqc was used to check the read quality at each step. Adaptor removal and quality filtering was performed using cutAdapt and BMTagger was used for host contamination removal (Love, Huber, & Anders, 2014; Martin, 2011). Quality controlled reads were passed on to MetaPhlAn 3 pipeline for taxonomy assignment.

Semi-Untargeted Stool Metabolomics and Data Processing:

The semi-targeted stool metabolome analysis was adopted from previously published studies (Groves et al., 2022; Mager et al., 2020; Rydzak et al., 2022). OMNImet.GUT samples were centrifuged for 10 min at 4000 × g at 4˚C, 100 µl of each supernatant was diluted with 100 uL of water. Metabolic analysis was performed on a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo-Fisher) coupled to a Vanquish™ UHPLC System (Thermo-Fisher). Chromatographical separation of metabolites was performed on Syncronis HILIC UHPLC column (2.1mm x 100mm x 1.7um, Thermo-Fisher) at the flow rate of 600uL/min using a binary solvent system: solvent A, 20mM ammonium formate pH 3.0 in mass spectrometry grade H_20 and solvent B, mass spectrometry grade acetonitrile with 0.1% formic acid (%v/v). Two chromatographic gradients are used simultaneously. The gradient on first pump was as follows: solvent B: 95% from 0 to 0.5 min; 95% to 5% from 0.5 to 3.5 min; and 5% from 3.5 to 4.5 min. The gradient on the second was 95% from 0 to 0.5 mins; 95% to 5% from 0.5 to 0.75 min; 5% from 0.75 to 2 min; 5% to 95% from 2 to 2.25 min; and 100% from 2.25 to 4.5 min. The mass spectrometer was run in negative full scan mode at a resolution of 240,000 scanning from 50-750m/z. Metabolite data was analyzed by El-MAVEN and MINT software packages (Clasquin, Melamud, & Rabinowitz, 2012; Melamud, Vastag, & Rabinowitz, 2010). Metabolites were identified by matching observed m/z signals (+/-10ppm) and chromatographic retention times to those observed from commercial metabolite standards (LMSLS™ Sigma-

Aldrich). Next, metabolites were quantified by comparison to an eight-point quantification curve of metabolite standards.

SCFAs were quantified as described previously (Bihan et al., 2022). In brief, OMNImet.GUT samples were centrifuged for 10 min at 4000 × g at 4˚C. 100 µl of each supernatant was then dispensed into 96-well plates. 10 µL of an ice-cold stable isotope-labeled SCFA internal standards (IS) solution, at a known concentration for all individual standard (acetic acid-1,2-13C2, propionic acid-13C, butyric acid-1,2-13C2, isobutyric-d7 acid, valeric-d9 acid and isovaleric-d9 acid) was added to each well, followed by aniline (5 µL) and EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, 5 µL) solutions (2.4 and 1.2 M, respectively). Samples were kept at 0˚C for 2 hours with regular shaking, then diluted with H2O/MeOH (50:50, v/v) prior to LC-MS/MS analysis. LC-MS/MS analysis was performed on a VanquishTM ultra high-performance liquid chromatography (UHPLC) system coupled to a TSQ QuantumTM Access MAX triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (HESI-II) probe. The UHPLC-MS platform was controlled by an XcaliburTM data system (Thermo Fisher Scientific). Chromatographic separation was achieved on a Hypersil GOLD TM C18 column (200 X 2.1 mm, 1.9 µm, Thermo Fisher Scientific) by using a binary solvent system composed of liquid chromatography–mass spectrometry grade water and methanol, both containing 0.1% (%v/v) formic acid and monitored with the mass spectrometer operating in positive ionization mode and selected reaction monitoring (SRM) mode. Electrospray ionization source conditions were as follows: spray voltage of 3000 V, vaporizer temperature of 325˚C, sheath gas of 35 psi, auxiliary gas flow of 10 (arbitrary units) and sweep gas flow of 2 (arbitrary units), capillary temperature of 275˚C. Data analyses, on the converted mzXML files, were conducted in El-MAVEN (Agrawal et al., 2019; Clasquin et al., 2012), and the absolute quantification of native SCFA concentration was based on the ^{12}C:IS signal intensity ratio and the respective known IS concentration.

For functional profiling, HUMAnN 3 was used to attain gene family and metabolic pathway abundances (i.e., MetaCyc, KO, eggnong) (Beghini et al., 2021).

2.7 – Data Cleaning and Imputation

Questionnaire and demographic data were examined for missing, outlier and duplicate data. To address missing data, the R package 'mice' (Multivariate Imputation by Chained

Equations) was used to perform Maximum Likelihood Estimation (MLE) imputation (Buuren & Groothuis-Oudshoorn, 2011). The 'mice' package applies an iterative algorithm to impute missing data, accounting for relationships among variables and preserving multivariate distribution. After imputation, the data were assessed using diagnostic plots, and comparisons of means and variances to evaluate the distribution.

2.8 – Data Analysis

For alpha diversity, several measures (i.e., observed species, Chao1, Simpson, and Shannon indices) were calculated to assess the richness and evenness of species within individual samples. Linear regression models and Spearman correlations were then used to explore associations with exposure variables such as hygiene scores and social contact.

Beta diversity was calculated using Aitchison distance as described by (Thomaz F. S. Bastiaanssen, 2022). For assessing changes in beta-diversity statistically, permutational multivariate analysis of variance (PERMANOVA) was performed on CLR transformed data, using Aitchison distance and beta-diversity was visualized using principal coordinate analysis (PCoA). This analysis was conducted to understand how variations in exposure to social and environmental factors, such as hygiene practices and social contact, might lead to distinct microbial community structures, as measured by beta diversity. We expected to see clustering of samples according to these exposures, which could indicate that these factors play a role in shaping the composition of the gut microbiome.

Correlations were performed using spearmen correlations. Corrections for multiple testing were done using the Benjamini-Hochberg method for bacterial taxa and metabolites, where an Adj. $p < 0.05$ was considered significant.

Both logistic and linear regression models were explored to assess the relationships between gut microbiome features (e.g., alpha diversity, specific taxa) and socioemotional outcomes as measured by the Ages & Stages Questionnaire: Social-Emotional (ASQ: SE) scores.

The R mediation package was used to process the data and assess mediation (Tingley, Yamamoto, Hirose, Keele, & Imai, 2014). As the primary outcome, scores on the ASQ:SE were assessed as a categorical variable (i.e., below cutoff, monitor and refer) and as continuous (i.e., overall score). Hygiene and social contact scores were assessed as exposure variables, while principal components to represent beta diversity were assessed as a mediator.

Metabolomic data were analyzed using MetaboAnalyst 5.0, a comprehensive online tool for metabolomic data processing and analysis (Pang et al., 2024). Concentration tables with detected metabolites and variables of interest were uploaded to MetaboAnalyst. The data were normalized and scaled to ensure comparability across samples. Pathway analysis was conducted to identify significant metabolic pathways associated with exposure and outcome variables. Pathway impact scores were calculated to determine the biological significance of the identified pathways (Chong, Wishart, & Xia, 2019).

2.9 – Covariate Measures

As discussed, a variety of factors can impact the infant microbiota composition, contributing to individual differences in the microbiome. Our analysis considered the inclusion of covariates, such as sex, breastfeeding status, antibiotic and medication use, delivery mode, and gestational age at birth (Levin et al., 2016; John Penders et al., 2006).

3.0 - Results

3.1 – Associations Between Measures of Alpha Diversity and Social Contact, Hygiene Practices

To address aim 1 and investigate if increased hygiene and reduced social contact are associated with reduced microbiome diversity, we performed a series of linear regression analyses and spearman correlations between alpha diversity indices and social contact as well as hygiene scores. Alpha diversity was assessed using indices of Chao1, Observed Species, Shannon, and Simpson.

The linear regression analyses revealed a significant positive association between the Chao1 index, a measure of community richness, and social contact. The initial model showed a positive association with a coefficient (β) of 1.884 ($p = 0.026$) and an adjusted R^2 of 0.013. This association remained significant after adjusting for covariates, showing an increased effect size ($\beta = 2.392$, $p = 0.004$) and improved model fit (Adjusted $R^2 = 0.134$). In contrast, no significant associations were found between hygiene scores and any measures of alpha diversity, including Chao1, Observed, Shannon, and Simpson indices.

The correlation between social contact and the Chao1 index was moderate ($r = 0.14$), and similar correlations were observed between social contact and other indices, such as Observed ($r = 0.14$) and Shannon ($r = 0.06$) (Figure 2). Hygiene scores as well as variables for living on a farm or with a furry pet had non-significant correlations with all alpha diversity measures.

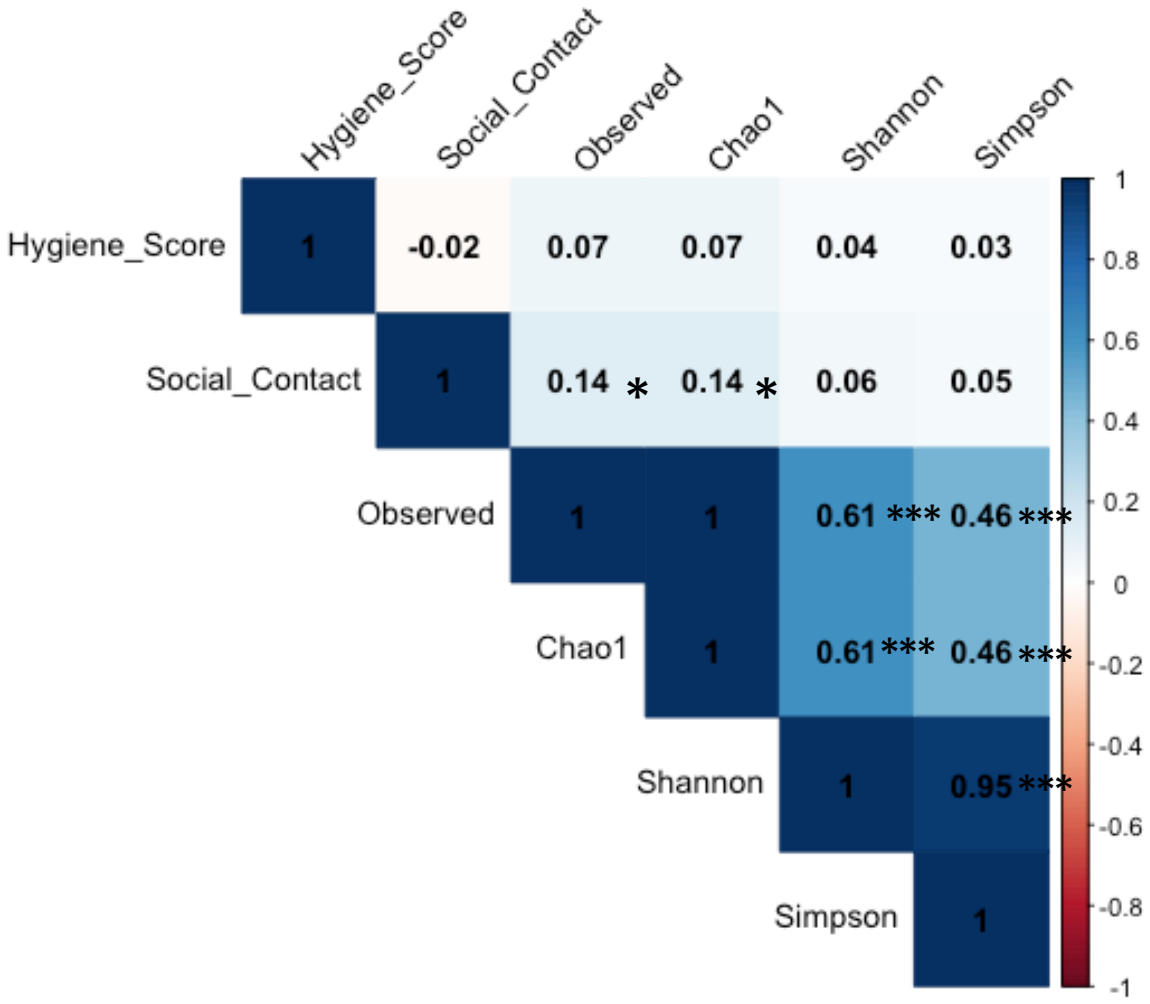

Figure 2. Correlation Matrix of Hygiene and Social Contact Scores with Diversity Indices. *The correlation matrix illustrates the relationships between hygiene scores, social contact scores, and alpha diversity indices (Observed Species, Chao1, Shannon, and Simpson). The color gradient represents the strength and direction of the correlations, with darker colors indicating stronger correlations. Significant correlations are marked with asterisks.*

3.2 - Effect of Hygiene Practices and Social Contact Scores on Beta Diversity

To further explore associations between exposure variables and microbiome composition, beta diversity of the samples was examined. Principal Component Analysis (PCA) was used to visualize the impact of scores (e.g., Hygiene, Social Contact, Living on a Farm, Presence of Furry Pet) and covariates known to impact the microbiome on beta diversity (Figure 3). The first two principal components (PCs) explained a significant portion of the variance, with PC1 accounting for 11.83% and PC2 for 7.07%. The biplot illustrates the influence of several covariates on the overall microbial composition.

The vectors on the biplot represent spearman correlations between each variable and the principal components (PC1 and PC2). The cluster patterns observed in the plot indicate variability in microbiome composition associated with these covariates. Samples positioned closer together suggest more similar microbial communities, whereas those further apart indicate greater dissimilarity.

The direction and magnitude of the correlation vectors highlight the influence of each variable on beta diversity. Notably, the Social Score exhibits a distinct and significant direction in the upper right quadrant. Other covariates, while also impactful, show varying directions and magnitudes of influence. Breastfeeding Status, Hygiene Score, Antibiotics Usage, and Delivery Method, among others, also contribute to the variability observed in microbiome composition.

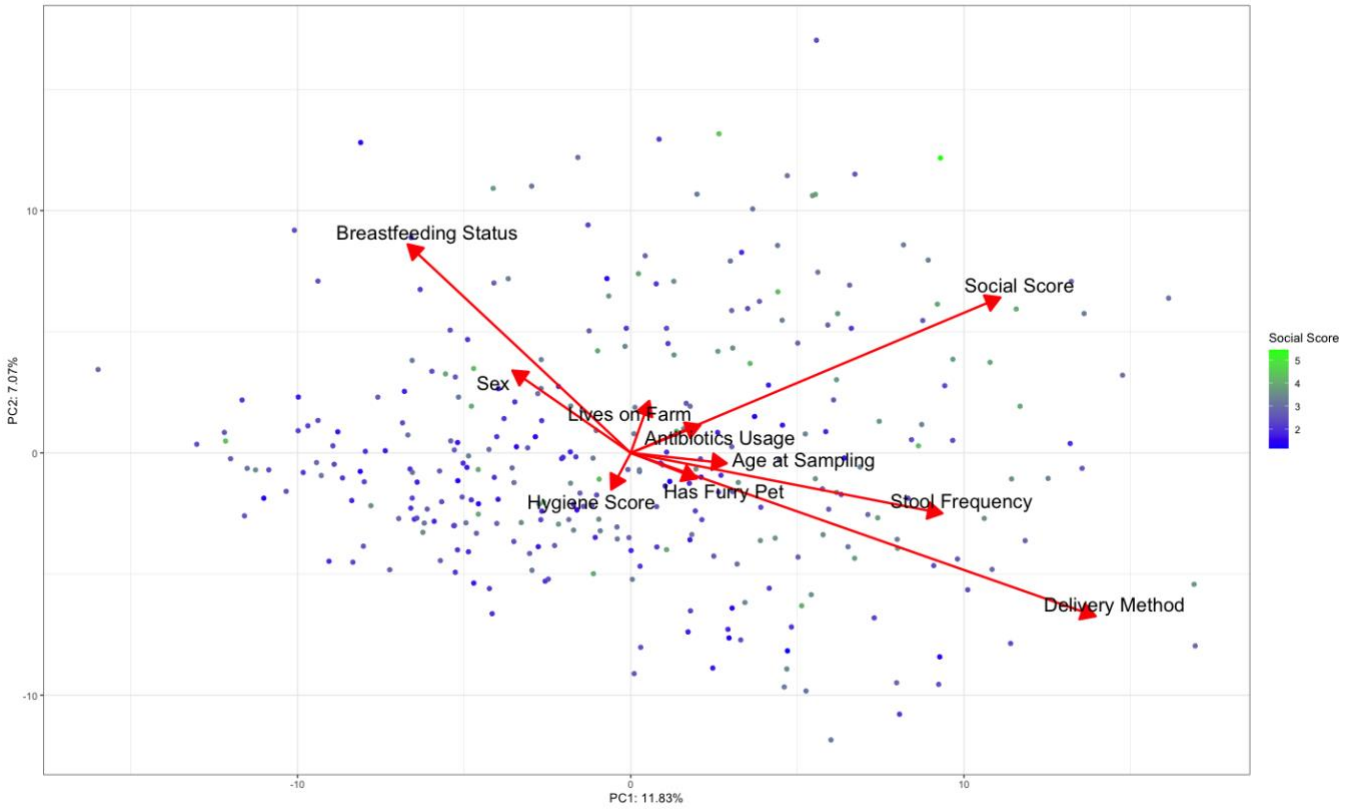

Figure 3: PCA Plot of Beta Diversity with Spearman Correlations of Social and Hygiene Variables. *The PCA plot shows the relationship between microbial taxa and beta diversity, with PC1 explaining 11.83% of the variance and PC2 explaining 7.07%. Arrows representing individual microorganisms' Spearman correlations have been magnified by a factor of 30x for visibility. Each point represents a sample, and the arrows indicate the direction and magnitude of the correlation between specific microbial taxa and the principal components. The color gradient represents the Social Score, with darker colors indicating higher scores.*

3.3 - Associations Between Taxa and Beta Diversity

A second PCA (Figure 4) was conducted focusing on microbial taxa to examine their associations with beta diversity (PC1 at 11.83% and PC2 at 7.07%). Several species of *Bifidobacterium*, including *Bifidobacterium bifidum* and *Bifidobacterium longum*, project towards the upper right quadrant, indicating a positive association with both PC1 and PC2. Other microbial taxa, such as *Enterococcus faecalis* and *Veillonella parvula*, show distinct positions on the biplot.

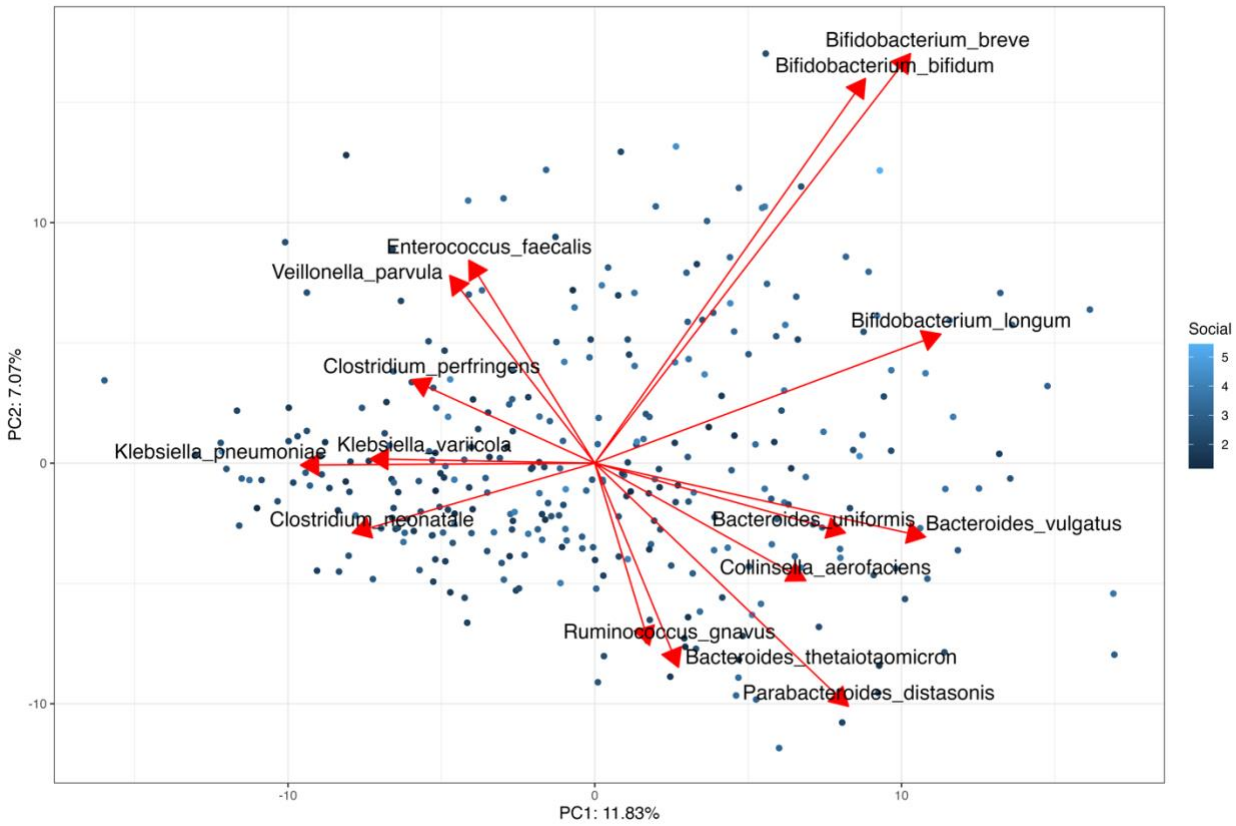

Figure 4. PCA Plot of Beta Diversity with Spearman Correlations of Individual Microorganisms. *Cluster patterns in the PCA plot indicate variability in microbiome composition associated with the evaluated covariates. Samples closer together suggest similar microbial communities, whereas samples further apart indicate greater dissimilarity. Note: arrows have been magnified by a factor of 30x for visibility, and only 15 correlations (of largest total magnitude) have been included for improved visibility.*

3.4 - PERMANOVA Analysis of Microbiome Variance

To evaluate the effect size and statistical significance of various factors on microbiome composition and variance, PERMANOVA analyses were performed to determine how much of the variance in the data each factor can explain (Table 4 – 8).

When assessing each score individually, only the Social Contact Score showed a significant effect on microbiome variance (Table 4). Specifically, the Social Contact Score explained 1.4% of the variance ($R^2 = 0.014$) with a significant F-statistic of 4.27 ($p = 0.001$). Other factors, including Hygiene Score, Presence of Furry Pet, and Living on a Farm, did not show significant effects on microbiome variance.

We then conducted a combined PERMANOVA including additional covariates (Table 8). The Social Contact Score remained significant, indicating an independent effect on microbial community composition not confounded by other covariates. Additionally, several other factors significantly influenced microbiome composition. Delivery Method explained 1.8% of the variance ($R^2 = 0.018$) with an F-statistic of 6.14 ($p = 0.001$). Breastfeeding status explained 2.3% of the variance ($R^2 = 0.023$) with an F-statistic of 4.43 ($p = 0.001$), and the presence of siblings explained 1.4% of the variance ($R^2 = 0.014$) with an F-statistic of 3.91 ($p = 0.001$). Stool Frequency explained 0.39% of the variance ($R^2 = 0.0039$) with an F-statistic of 6.55 ($p = 0.001$), and Age at Sampling explained 0.83% of the variance ($R^2 = 0.0083$) with an F-statistic of 2.29 ($p = 0.002$). Other variables did not show significant effects.

Table 4. Individual PERMANOVA for Social Contact Score.

	R^2	F	Pr(>F)
Social Contact Score	0.014	4.27	**0.001**
Residual	0.986		
Total	1.000		

Table 5. Individual PERMANOVA for Hygiene Score.

	R^2	F	Pr(>F)
Hygiene Score	0.0034	1.04	0.38
Residual	0.997		
Total	1.000		

Table 6. Individual PERMANOVA for Presence of Furry Pet Variable.

	R^2	F	Pr(>F)
Presence of Furry Pet	0.0045	1.39	0.082
Residual	0.995		
Total	1.000		

Table 7. Individual PERMANOVA for Living on a Farm Variable.

	R^2	F	Pr(>F)
Living on a Farm	0.0031	0.95	0.51
Residual	0.997		
Total	1.000		

Table 8. Combined PERMANOVA With Addition of Covariates.

	R2	F	Pr(>F)
Social Contact Score	0.014	4.32	**0.001**
Hygiene Score	0.0040	1.17	0.24
Presence of Furry Pet	0.0043	1.34	0.11

Live on a Farm	0.0037	0.98	0.45
Delivery method	0.018	6.14	**0.001**
Breastfeeding	0.023	4.43	**0.001**
Siblings	0.014	3.91	**0.001**
Antibiotics	0.0040	1.26	0.16
Stool Frequency	0.0039	6.55	**0.001**
Age at Sampling	0.0083	2.29	**0.002**
Residual	0.90		
Total	1		

3.5 - Taxa Associated with Social Contact and Hygiene Practices

To further investigate the associations between microbiome composition and our scores of interest, specific taxa correlated with Social Contact (Table 9, Figure 5) and Hygiene practices (Table 10, Figure 6) were examined.

Social Contact: Several species of *Bifidobacterium* showed a significant positive correlation with Social Contact scores. *Bifidobacterium breve* had the highest correlation (r = 0.30, p = 6.25E-08, adjusted p = 4.25E-06), indicating that higher social contact scores were associated with a higher abundance of this bacterium. Similarly, *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium longum* also showed significant positive correlations with Social Contact. Other taxa, such as *Haemophilus parainfluenzae* and *Klebsiella pneumoniae*, exhibited significant negative correlations with Social Contact.

Hygiene Practices: In contrast to social contact, hygiene practices had a less pronounced effect on specific microbial taxa. However, several taxa did show significant associations. *Faecalibacterium prausnitzii* and *Eubacterium rectale* remained significant after adjusting for multiple testing, with *Faecalibacterium prausnitzii* showing a positive correlation (r = 0.19, p = 0.00078, adjusted p = 0.046) with hygiene scores. *Eubacterium rectale* also showed a significant positive correlation (r = 0.18, p = 0.0014, adjusted p = 0.046).

Table 9: Taxa Associated with Social Contact.

Species	Correlation	p Value	Adjusted p Value
Bifidobacterium breve	0.30	6.25E-08	**4.25E-06**
Bifidobacterium bifidum	0.20	0.00040	**0.010**
Bifidobacterium pseudocatenulatum	0.20	0.00046	**0.010**
Bifidobacterium longum	0.19	0.00072	**0.012**
Haemophilus parainfluenzae	-0.17	0.0023	**0.032**
Faecalibacterium prausnitzii	0.17	0.0036	**0.041**
Bacteroides dorei	0.16	0.0043	**0.041**
Alistipes putredinis	0.16	0.0064	0.054
Prevotella copri	0.15	0.0073	0.055
Clostridium neonatale	-0.15	0.0092	0.062
Bacteroides vulgatus	0.14	0.012	0.071

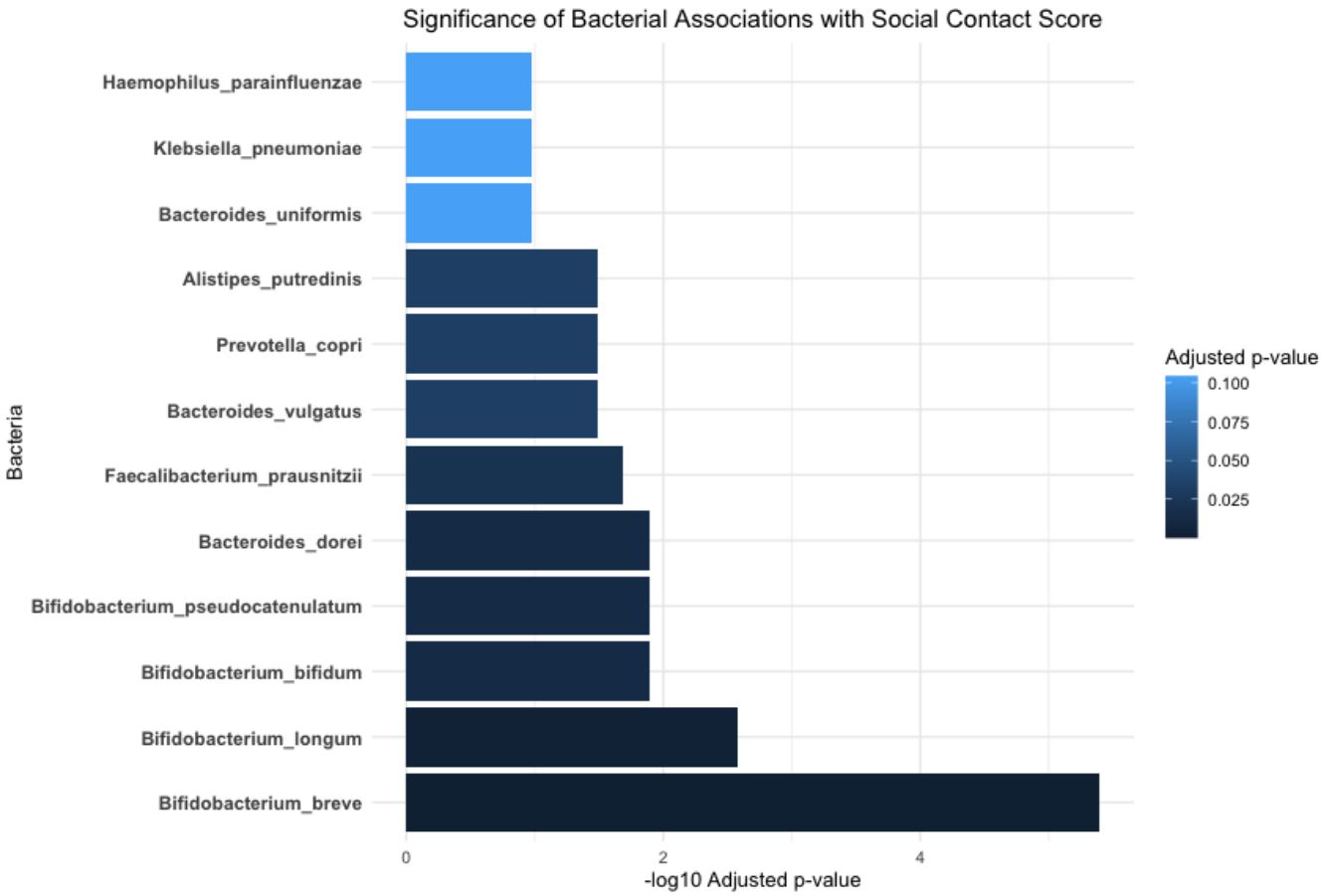

Figure 5. Significance of Bacterial Presence in Microbiome Associated with Social Contact Score. *The x-axis represents the -log10 adjusted p-values, indicating the strength of the association, while the y-axis lists the bacterial taxa. The color gradient indicates the adjusted p-value, with darker colors representing more significant associations.*

Table 10: Taxa Associated with Hygiene Practices Score.

Species	Correlation	p Value	Adjusted p Value
Faecalibacterium prausnitzii	0.19	0.00078	**0.046**
Eubacterium rectale	0.18	0.0014	**0.046**
Fusicatenibacter saccharivorans	0.16	0.0059	0.13
Alistipes putredinis	0.14	0.016	0.28
Prevotella copri	-0.11	0.062	0.59

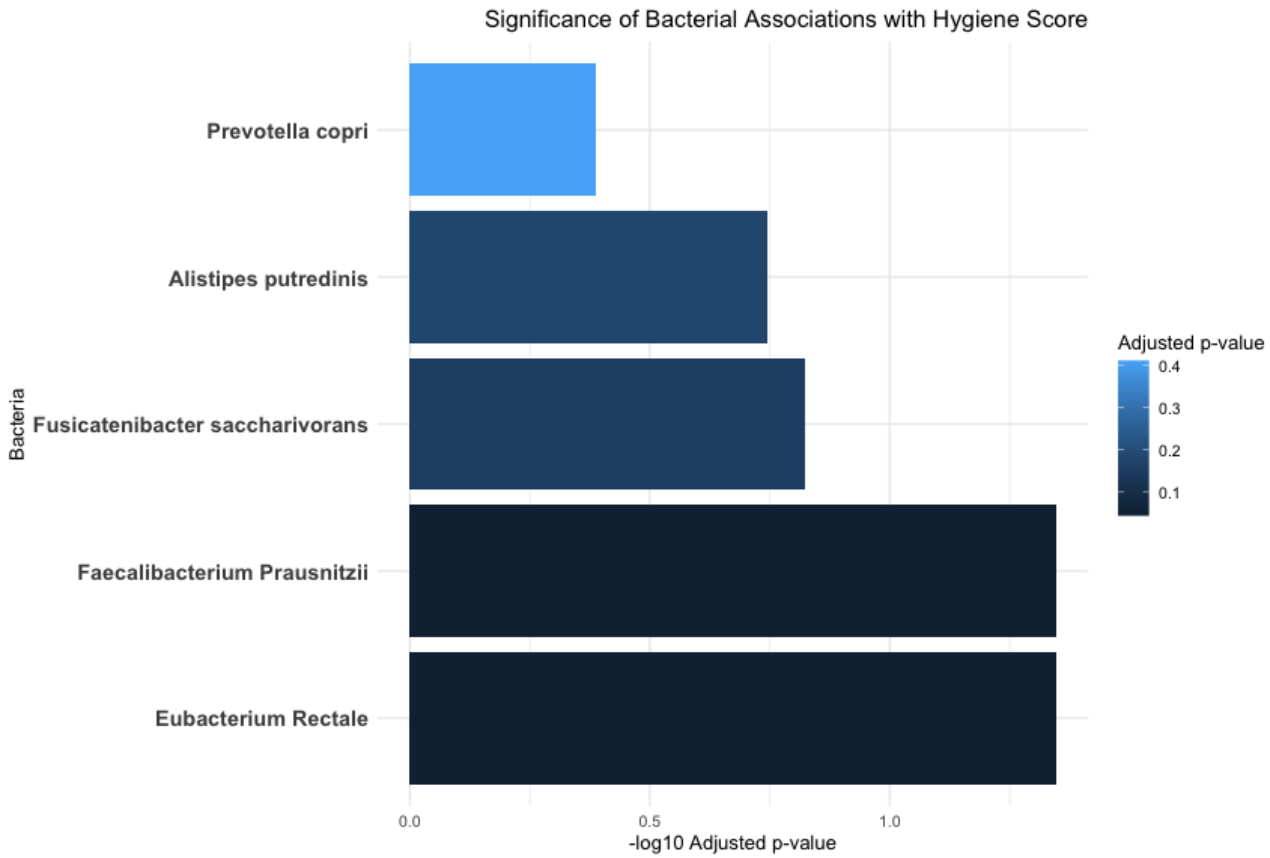

Figure 6. Significance of Bacterial Presence in Microbiome Associated with Hygiene Score. *The x-axis represents the -log10 adjusted p-values, indicating the strength of the association, while the y-axis lists the bacterial taxa. The color gradient indicates the adjusted p-value, with darker colors representing more significant associations.*

3.6 - Metabolic Pathway Associations with Social Contact and Hygiene Practices

Further analysis using MetaboAnalyst revealed significant metabolic pathways associated with social contact (Table 11) and hygiene scores (Table 12). Social contact significantly influenced a broad array of metabolic functions. The pathway with the strongest association was Alanine, Aspartate, and Glutamate Metabolism ($p < 0.001$). Other highly significant pathways included Arginine Biosynthesis ($p < 0.001$) and Pentose and Glucuronate Interconversions ($p < 0.001$). Approximately 30 pathways were significantly associated with social contact scores, with varying impact scores.

Hygiene practices showed significant associations with fewer metabolic pathways. The most significant pathways were Butanoate Metabolism ($p = 0.001$) and Beta-Alanine Metabolism ($p = 0.001$), which remained significant after FDR correction.

Table 11. Metabolic Pathways Significantly Associated with Social Contact Scores.

* Only contains pathways that passed significance threshold of $p < 0.05$ after correction for multiple testing

Pathway	Matched Compounds	Raw p	FDR	Impact
Alanine, aspartate, and glutamate metabolism	8/28	3.46E-07	1.02E-05	0.47
Nitrogen metabolism	1/6	3.91E-07	1.02E-05	0
Arginine biosynthesis	8/14	1.41E-06	2.44E-05	0.37
Pentose and glucuronate interconversions	1/18	2.03E-06	2.64E-05	0.078
Aminoacyl-tRNA biosynthesis	16/48	4.36E-06	4.53E-05	0
Glyoxylate and dicarboxylate metabolism	4/32	5.56E-06	4.82E-05	0.13
D-Glutamine and D-glutamate metabolism	3/6	6.51E-06	4.84E-05	0
Galactose metabolism	2/27	1.25E-05	8.15E-05	0.090
Primary bile acid biosynthesis	7/46	5.06E-05	0.00029	0.041
Taurine and hypotaurine metabolism	5/8	0.00040	0.0021	0.71
Glycine, serine and threonine metabolism	5/33	0.00065	0.0031	0.29

Biosynthesis of unsaturated fatty acids	2/36	0.0010	0.0045	0
Terpenoid backbone biosynthesis	1/18	0.0012	0.0049	0.11

Table 12. Metabolic Pathways Significantly Associated with Hygiene Practices Scores.

* Only contains pathways that passed significance threshold of $p < 0.05$ after correction for multiple testing

Pathway	**Matched Compounds**	**Raw p**	**FDR**	**Impact**
Butanoate metabolism	5/15	0.0010	0.035	0.11
beta-Alanine metabolism	5/21	0.0013	0.035	0.16

3.7 - Influence of Social Contact and Hygiene Practices on Microbial Presence and Abundance

To address aim 2 and investigate the effect of social contact on the presence or absence of specific microbes, the sample was divided into two groups based on the median social contact score: high contact and low contact groups. The proportion of each group that had specific bacteria detected in the samples was calculated and these proportions were compared between the two groups (Figure 7, Figure 8).

The analysis revealed that *Bifidobacterium breve* was the most notably different species, present in about 60% of the high contact samples but only in approximately 30% of the low contact samples, resulting in an absolute score difference of 0.297. Other species with significant differences included *Bacteroides dorei, Bacteroides vulgatus,* and *Bifidobacterium bifidum*.

A similar analysis was conducted to understand the impact of hygiene practices on the microbiome. Fewer significantly different species were found to be associated with hygiene practices compared to social contact. Only two species showed an absolute difference above 0.1: *Faecalibacterium prausnitzii* (0.113) and *Eubacterium rectale* (0.12).

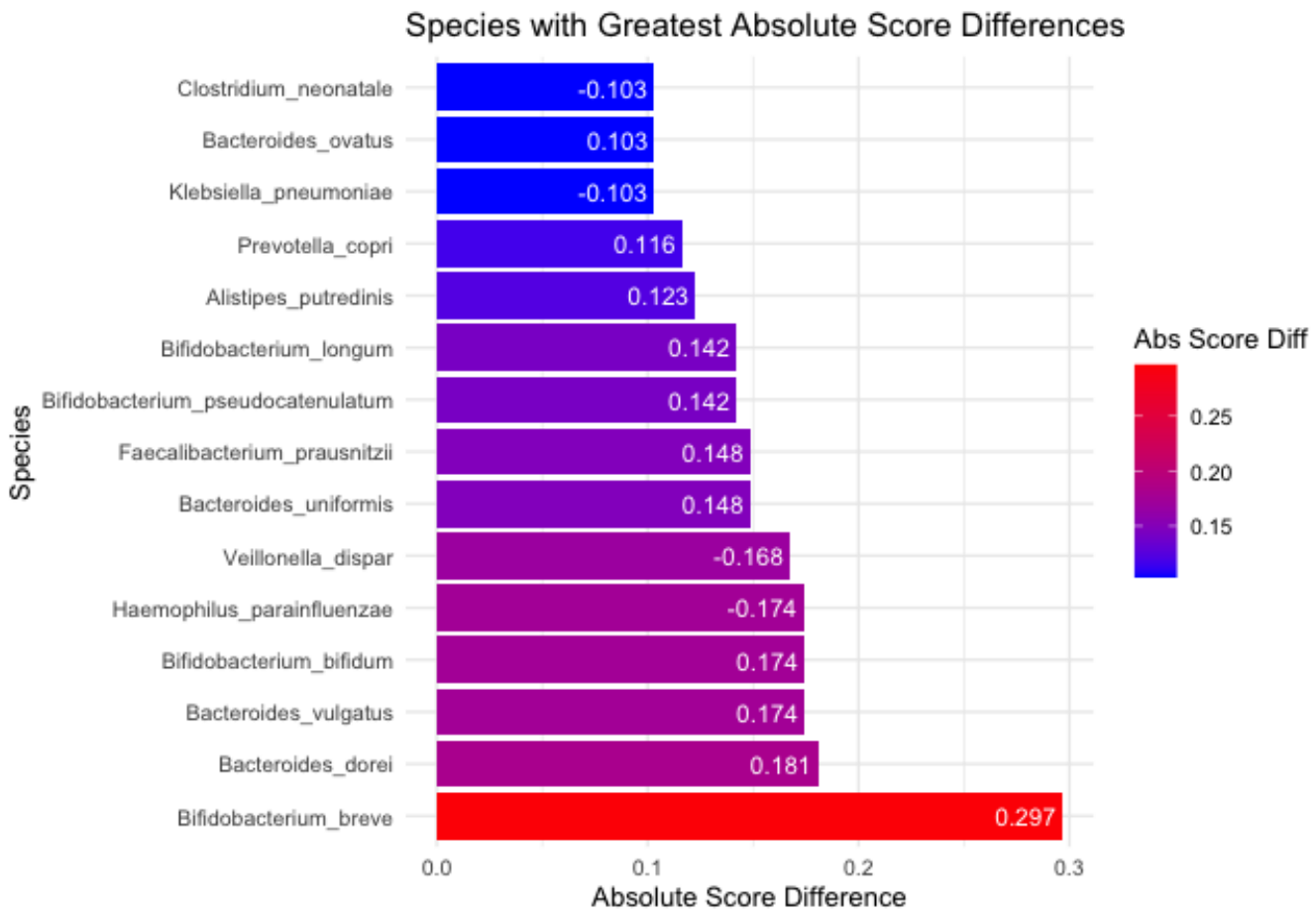

Figure 7. Social Contact and Presence of Microbes Across Samples. *The x-axis represents the absolute score difference, indicating the difference in the proportion of each group that harbors specific bacteria. The y-axis lists the bacterial species, with the color gradient indicating the magnitude of the absolute score difference.*

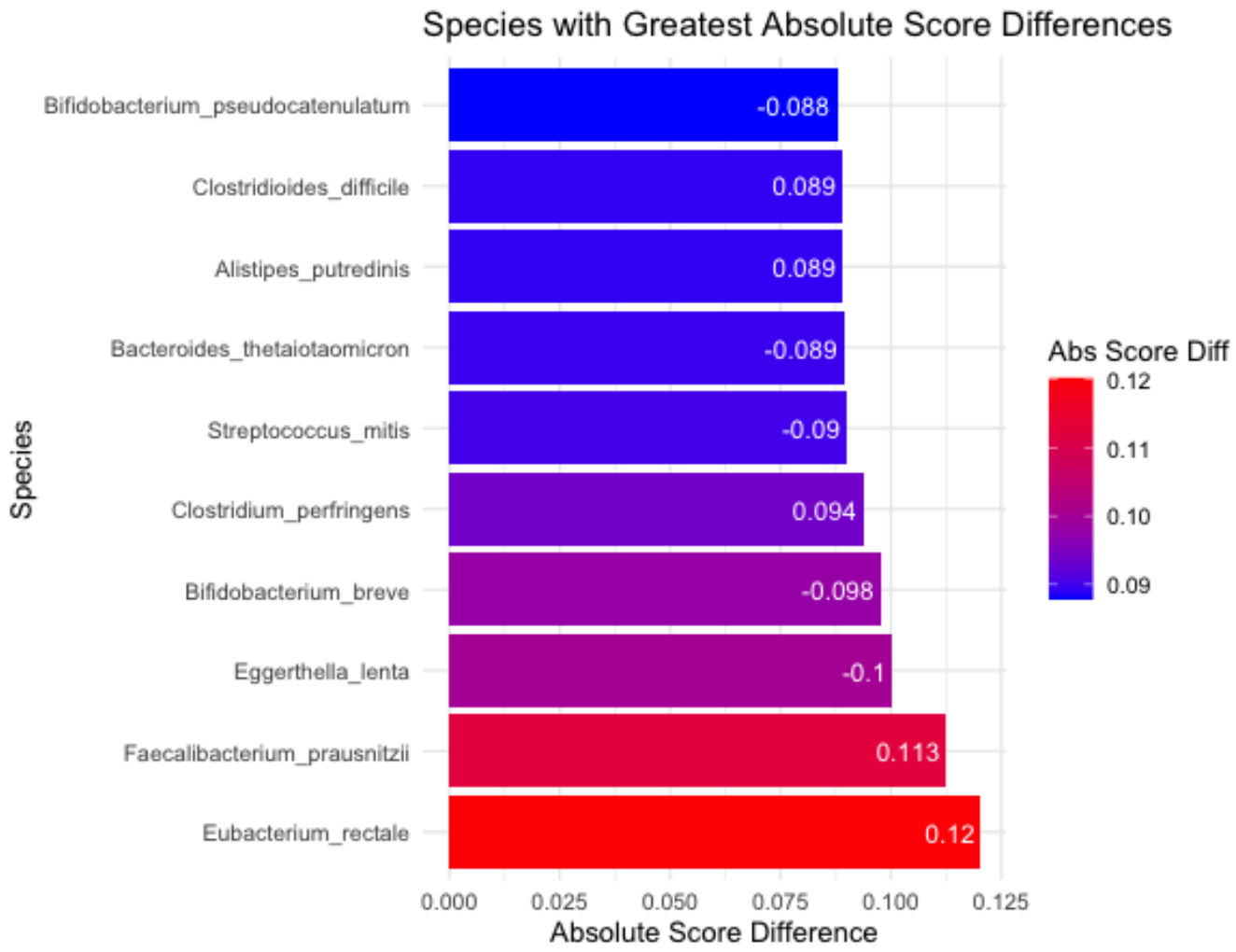

Figure 8. Hygiene Practices Score and Presence of Microbes Across Samples. *The x-axis represents the absolute score difference, indicating the difference in the proportion of each group that harbors specific bacteria. The y-axis lists the bacterial species, with the color gradient indicating the magnitude of the absolute score difference.*

3.8 - Alpha Diversity and ASQ: SE Scores

To address aim 3 and determine if features of gut microbiome associate with and predict socioemotional outcomes, we first investigated the relationship between alpha diversity and the ASQ:SE scores using linear regression analyses. The breakdown of ASQ: SE scores into developmental screening categories is depicted in Table 13. Alpha diversity was measured using the Simpson index, which encapsulates both the richness and evenness of species in a community as it represents the probability that two individuals randomly selected from a sample will belong to the same species (Liu et al., 2020).

The analysis revealed a significant positive association between the Simpson alpha diversity index and raw ASQ:SE scores ($\beta = 0.0014$, $p = 0.023$, Adjusted $R^2 = 0.015$). The model's fit was slightly improved by the inclusion of covariates ($\beta = 0.0016$, $p = 0.022$, Adjusted $R^2 = 0.068$).

No significant associations were found for the ASQ: SE cutoff scores, and there were no significant Spearman correlations for either score type.

Table 13. Spread of ASQ:SE Results at 12 Months of Age.

*87 samples did not have a completed ASQ: SE score

	N	%
Below cutoff - development is not at risk	632	80.4 %
Monitor	106	13.4 %
Refer	48	6.1 %

3.9 - Effect of Scores and Covariates on Beta Diversity with Addition of ASQ: SE Scores

We conducted a Principal Component Analysis (PCA) to assess the impact of various factors on beta diversity. The PCA plot (Figure 9) displays the first two principal components (PC1 and PC2), which explain 11.83% and 7.07% of the variance, respectively. Vectors on the biplot represent Spearman correlations between each variable and the principal components, magnified for clarity.

The plot indicates that the Social Score is associated with a shift in microbiome composition as measured by beta diversity, projecting into the upper right quadrant. In contrast, the Ages & Stages Questionnaires: Social-Emotional (ASQ:SE) score exhibits an opposite directional effect on beta diversity compared to social contact, with higher ASQ:SE scores (associated with greater socioemotional problems) projecting into the bottom left of the quadrant.

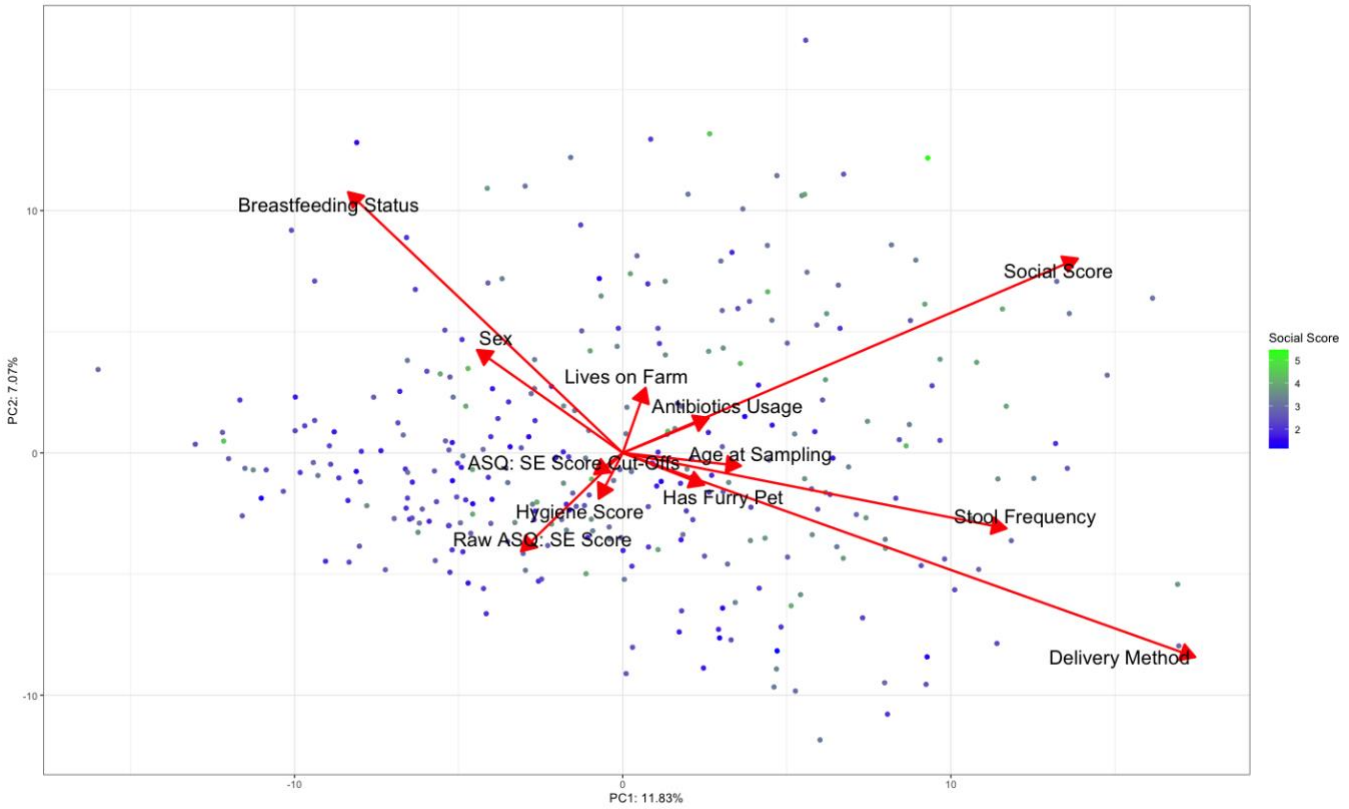

Figure 9. PCA Plot of Beta Diversity with Spearman Correlations of Social and Hygiene Variables with Addition of ASQ: SE Variables. *The PCA plot shows the relationship between microbial taxa and beta diversity, with PC1 explaining 11.83% of the variance and PC2 explaining 7.07%. Arrows representing individual microorganisms' Spearman correlations have been magnified by a factor of 30x for visibility. Each point represents a sample, and the arrows indicate the direction and magnitude of the correlation between specific microbial taxa and the principal components. The color gradient represents the Social Score, with darker colors indicating higher scores.*

3.10 - PERMANOVA Analysis for ASQ: SE Scores

We conducted a PERMANOVA to investigate the variance explained by the Ages & Stages Questionnaires: Social-Emotional (ASQ: SE) scores on microbiome composition (Tabe 14, Table 15). The analysis revealed that the raw ASQ: SE scores significantly explained a small portion of the variance, with an R^2 of 0.0055 and an F-statistic of 1.53 ($p = 0.035$). When the ASQ: SE cutoff scores were analyzed separately, they did not show significant results ($R^2 = 0.0097$, $F = 1.34$, $p = 0.0539$).

To further refine our model, Social Contact was included as an additional variable with each analysis (Table 16, Table 17). This did not appear to affect the significance or explanatory power of the raw ASQ: SE scores ($R^2 = 0.0055$, $F = 1.55$, $p = 0.036$). However, the inclusion of social contact in the model improved the significance of Cutoff scores ($R^2 = 0.010$, $F = 1.68$, $p = 0.020$).

Table 14. Individual PERMANOVA Analyses for Raw ASQ:SE Scores.

	R^2	F	Pr(>F)
Raw ASQ: SE Score	0.0055	1.53	**0.035**
Residual	0.99		
Total	1.000		

Table 15. Individual PERMANOVA Analyses for Cutoff ASQ:SE Scores

	R^2	F	Pr(>F)
ASQ: SE Cutoff Score	0.0097	1.34	0.054
Residual	0.99		
Total	1.000		

Table 16. PERMANOVA Analysis for Raw ASQ:SE Score with Addition of Social Contact Score.

	R^2	F	Pr(>F)
Raw ASQ: SE Score	0.0055	1.55	**0.036**
Social Contact Score	0.016	4.35	**0.001**
Residual	0.98		
Total	1.000		

Table 17. PERMANOVA Analysis for Cutoff ASQ:SE Score with Addition of Social Contact Score.

	R^2	F	Pr(>F)
ASQ: SE Cutoff Score	0.010	1.68	**0.020**
Social Contact Score	0.015	4.33	**0.001**
Residual	0.98		
Total	1.000		

3.11 - Taxa Associations with ASQ: SE Scores

Next, it was investigated which microbial taxa are positively or negatively associated with Ages & Stages Questionnaires: Social-Emotional (ASQ: SE) scores (Table 18, Table 19). After correcting for multiple testing, we found that *Streptococcus salivarius* was significantly associated with lower raw ASQ: SE scores (adjusted $p = 0.049$). Lower ASQ: SE scores are indicative of better social-emotional development. Although several other associations (with *Clostridium neonatale, Klebsiella oxytoca,* and *Varibaculum cambriense*) were identified, they did not reach statistical significance after correction for multiple testing.

Similarly, taxa like *Escherichia coli, Clostridium innocuum, Ruminococcus gnavus, Veillonella dispar, Bifidobacterium adolescentis,* and *Clostridioides difficile* were found to have initial associations to cutoff ASQ: SE scores. However, none of these associations remained significant after correcting for multiple testing, with adjusted p-values all above 0.22.

Table 18. Raw ASQ: SE Scores Correlated with Bacterial Species.

Species	Correlation	p Value	Adjusted p Value
Streptococcus salivarius	-0.20	0.00072	**0.049**
Clostridium neonatale	0.16	0.0062	0.17
Klebsiella oxytoca	-0.16	0.0073	0.17
Varibaculum cambriense	-0.12	0.049	0.72

Table 19. Cutoff ASQ: SE Scores Correlated with Bacterial Species.

Species	Correlation	p Value	Adjusted p Value
Escherichia coli	0.18	0.0033	0.22
Clostridium innocuum	0.15	0.012	0.34
Ruminococcus gnavus	0.15	0.015	0.34
Veilonella dispar	0.14	0.020	0.34
Bifidobacterium adolescentis	0.13	0.027	0.35
Clostridioides difficile	0.13	0.031	0.35

3.12 - Metabolic Pathway Associations with Raw and Cut-off ASQ:SE Scores

The metabolomic analysis was again conducted using MetaboAnalyst to explore the associations between metabolic pathways and Ages & Stages Questionnaires: Social-Emotional (ASQ: SE) scores (Table 20, Table 21). Both the raw ASQ:SE scores (treated as a continuous variable) and the cutoff scores (treated as a categorical variable) were analyzed to identify significant pathways.

The analysis identified the Ascorbate and Aldarate Metabolism pathway as significantly associated with both raw and cutoff ASQ: SE scores. This pathway remained significant even after correcting for multiple testing in its association with the raw score ($p = 0.037$, impact = 0), with the driving metabolite being L-Gulono-1,4-lactone.

Table 20. Metabolic Pathways Significantly Associated with raw ASQ:SE Scores.

*Table includes all results with a raw p-value under $p < 0.05$ threshold significance

Pathway	Matched Compounds	p-value	FDR	Impact
Ascorbate and aldarate metabolism	1/9	7.41E-04	**0.037**	0
Thiamine metabolism	1/7	0.012	0.21	0
Glycine, serine and threonine metabolism	6/33	0.018	0.21	0.52
Tryptophan metabolism	8/41	0.021	0.21	0.43
Arginine and proline metabolism	7/36	0.021	0.21	0.35
Glutathione metabolism	3/28	0.039	0.33	0.09

Table 21. Metabolic Pathways Significantly Associated with cutoff ASQ:SE Scores.

*Table includes all results with a raw p-value under $p < 0.05$ threshold significance

Pathway	Matched Compounds	p-value	FDR	Impact
Ascorbate and aldarate metabolism	1/9	0.0015	0.074	0
Tryptophan metabolism	8/41	0.020	0.36	0.43
Glycine, serine and threonine metabolism	6/33	0.028	0.36	0.52
Arginine and proline metabolism	7/36	0.034	0.36	0.35
Glycerolipid metabolism	2/16	0.036	0.36	0.28

3.13 - Association of Social Contact & Hygiene on ASQ: SE

To address aim 4 and investigate the potential indirect effects of social contact and hygiene habits on socioemotional development with a focus on mediation by the microbiome or metabolome, a correlation analysis was first conducted. This involved performing correlations and linear regressions between social contact scores and Ages & Stages Questionnaires: Social-Emotional (ASQ:SE) scores.

The analysis did not find any significant associations between social contact scores and ASQ: SE scores. Correlation analysis yielded p-values of 0.52 for raw scores and 0.17 for cutoff scores, indicating no significant bivariate effect. Similarly, hygiene scores did not show significant correlations with ASQ:SE scores, with p-values of 0.37 and 0.22 for raw and cutoff scores, respectively. Linear regression analysis also confirmed the absence of significant relationships between these variables (Figure 10).

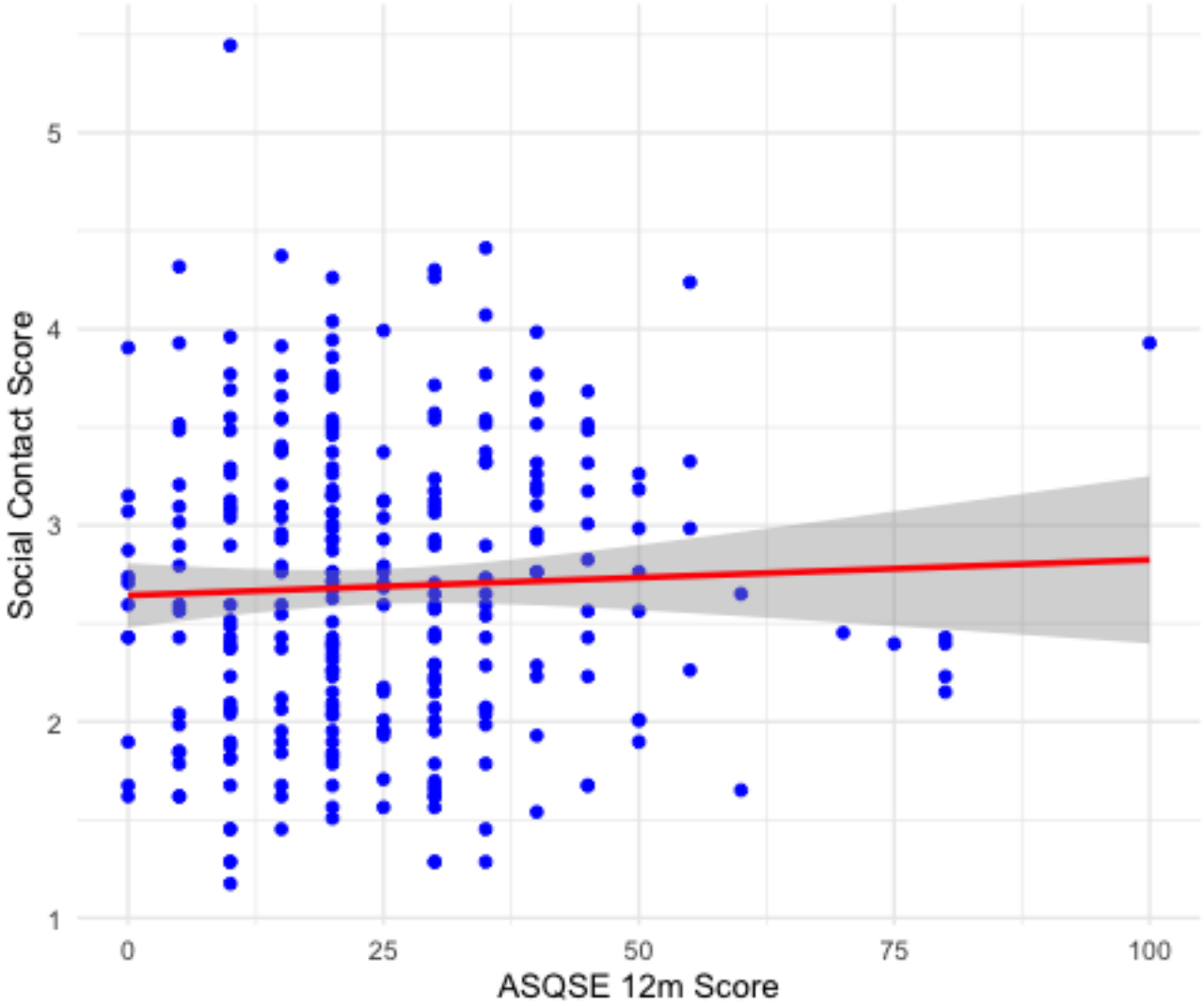

Figure 10. Relationship Between Social Contact Score and Raw ASQ:SE 12-Month Score. *The scatter plot shows the relationship between social contact scores and ASQ: SE 12-month scores. Each blue dot represents an individual sample, and the red line indicates the linear regression fit with a 95% confidence interval shaded in gray.*

3.14 - Mediation Analysis of Social Contact, Gut Microbiome, and ASQ: SE Scores

Despite the absence of a significant direct effect of social contact on socioemotional development, a mediation analysis was still conducted to test whether the gut microbiome mediates this relationship as the analysis was part of the predetermined research plan.

Though multiple gut microbiome features had associations with ASQ: SE scores, no distinct overlaps were found between ASQ: SE scores and social contact for individual taxa or alpha diversity measures. However, the opposite directional effects of social contact and raw ASQ:SE scores on beta diversity prompted the hypothesis that beta diversity might serve as a mediating variable.

Mediation analysis was performed using the first two principal components (PC1 and PC2) from the PCA of beta diversity as mediators. The analysis revealed that the Average Causal Mediation Effect (ACME) for PC1 was significant ($p = 0.044$), suggesting a statistically significant mediation effect (Figure 11). The Average Direct Effect (ADE) for PC1 was not significant ($p = 0.18$), confirming that the direct effect of social contact on socioemotional outcomes is not statistically significant after accounting for the mediating effect of the gut microbiome.

For PC2, both the ACME ($p = 0.12$) and ADE ($p = 0.16$) were not significant.

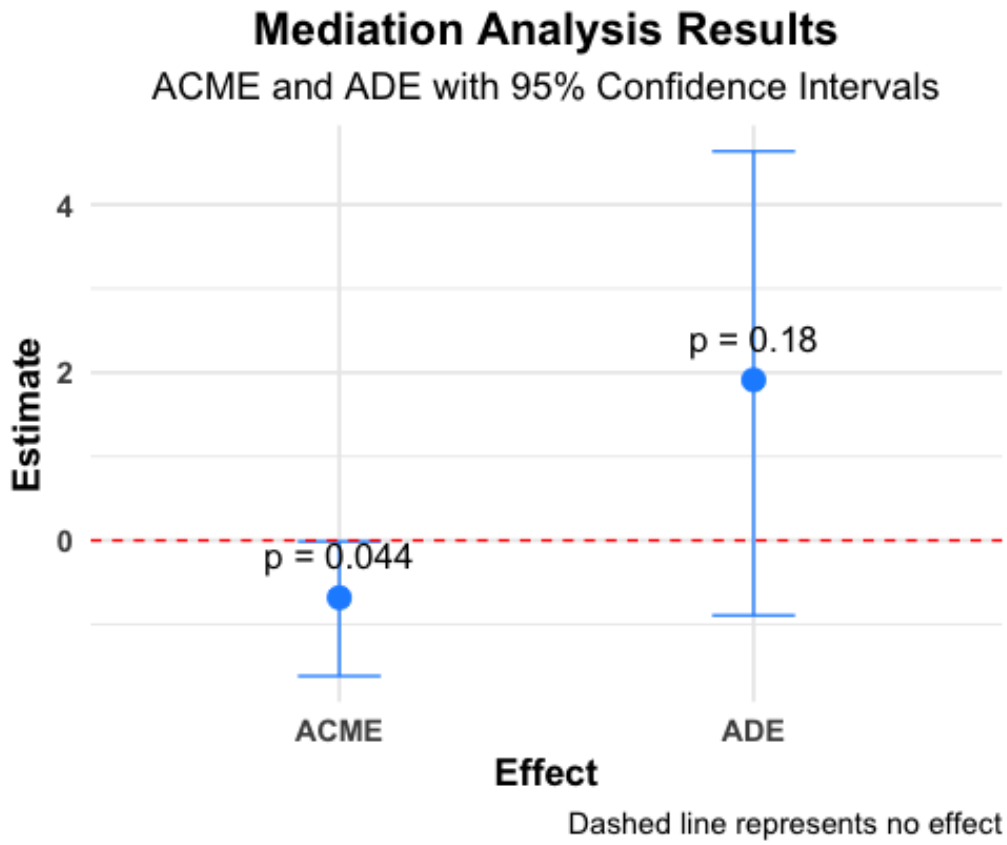

Figure 11. Mediation Analysis Results. *Principal component analysis (PCA) of beta diversity was used to derive the first two principal components (PC1 and PC2) as potential mediators. Each point represents the effect estimate with 95% confidence intervals, and the dashed line represents no effect.*

4.0 – Discussion

The present study examined the impact of increased hygiene practices and reduced social contact due to COVID-19 measures on the gut microbiome and socioemotional development in infants during their first year of life. The findings strongly support the hypothesis that changes in social contact are associated with the composition and diversity of the gut microbiome in infants. Comparatively, the effect of hygiene practices on the microbiome was less robust than expected. Social contact was found to have a notable influence on both alpha and beta diversity, as well as on the presence of specific beneficial microbial taxa such as *Bifidobacterium* species. However, the evidence linking these changes in the gut microbiome to socioemotional development at 1 year of age remains limited and requires further investigation.

4.1 – Associations of Social Contact and Hygiene Practices with Measures of Microbiome Diversity and Variance

Aim 1: Associations of Hygiene Practices and Social Contact with Gut Microbiome Diversity, Variance and Taxa

The first aim of the study was to investigate if increased hygiene and reduced social contact are associated with microbiome features, particularly measures of diversity and variance. Given the heightened hygiene practices and reduced social interactions during the COVID-19 pandemic, we expected these factors to significantly alter the gut microbiome's diversity and composition. Previous research suggested that reduced microbial exposure due to increased hygiene and reduced social contact would lead to lower diversity in the gut microbiome (Finlay et al., 2021); (Joann Romano-Keeler, Jilei Zhang, & Jun Sun, 2021).

Using several measures of alpha diversity (Observed species, Chao1, Simpson and Shannon), we attempted to determine associations between alpha diversity and our exposure variables through linear regression models and spearman correlations. The analysis revealed no significant associations between hygiene scores, living on a farm or having a furry pet and measures of alpha diversity. The lack of significant associations between hygiene practices and microbial species richness may indicate that hygiene practices, as they were measured in this study, did not independently influence the diversity of the infant microbiome.

However, social contact positively associated with microbial richness, as measured by the Chao1 index (and observed species), with a significant association ($\beta = 1.884$, $p = 0.026$;

adjusted $R^2 = 0.013$) (Figure 2). This suggests that increased social contact may contribute to greater microbial richness in infants. A possible mechanism based on the pattern of the findings are that social interactions facilitate transfer of a diverse array of microbes to the infant's gut, contributing to greater number of species observed. At three months of age, infants primarily engage in social contact through physical interactions with their caregivers and close family members. These interactions can often involve touching, hugging and kissing, and as caregivers are frequently in physical contact with infants there are many opportunities for microbial transfer through skin to skin contact or transfer of the oral microbiome (Ferretti et al., 2018). Though at three months direct feeding with shared utensils is less common, microbes from saliva can still be transferred during feeding or by cleaning pacifiers with the mouth. Physical play activities such as tummy time can also contribute to environmental microbe exposure (Hewitt, Kerr, Stanley, & Okely, 2020). Studies have shown that social interactions, such as those within households or communities, lead to significant microbial strain-sharing among individuals, increasing microbial diversity (Dill-McFarland et al., 2019).

The Principal Component Analysis (PCA) suggested that social contact may also influence beta diversity, with the social contact vector markedly directed towards the upper right quadrant (with specific *Bifidobacterium* taxa showing similar directional effects), indicating positive relationships with both PC1 and PC2 (Figure 3). This indicates a distinct association between social contact and variation in microbiome composition. Similar to results with alpha diversity, hygiene scores, living on a farm, or presence of a furry pet did not show the same magnitude of effect on beta diversity. The absence of a strong effect of hygiene practices on beta diversity is notable given the initial hypothesis that hygiene would have a substantial impact. This finding suggests that the role of hygiene practices as measured in our study for shaping the gut microbiome may be less pronounced compared to social contact during early infancy. It is also important to consider that the hygiene practices assessed in this study were influenced by the circumstances of the COVID-19 pandemic, during which increased sanitization and reduced microbial exposure were widespread (Byrne et al., 2023; J. Romano-Keeler et al., 2021). These pandemic-related changes could have created a unique context where the expected impact of hygiene on the microbiome might differ from pre-pandemic patterns, potentially explaining the weaker effect we observed.

To further explore how the exposure variables related to microbiome variance, PERMANOVA analyses were performed to determine what amount of variance in the microbiome was explained by each variable. Social contact was the only individual variable to significantly explain variance in the microbiome composition ($R^2 = 0.014$, $p = 0.001$). The R^2 and p-value for social contact scores did not change in the combined analysis, which could indicate that social contact has an independent effect on the microbial community composition that is not confounded by other variables in the analysis. Covariates such as delivery method, breastfeeding, presence of siblings, stool frequency, and age at sampling also showed significant effects. In the combined analysis, social contact had an effect size comparable to that of breastfeeding. This indicates that, similar to the documented benefits of breastfeeding on gut microbiome composition, social contact plays a crucial role in shaping the microbial environment in early infancy (B. J. Kelly et al., 2015) (Mueller et al., 2015).

Previous research shaped our prediction that hygiene practices would have a large effect on the gut microbiome (Romagnani, 2004; Wold, 1998). A 2018 study by M. H. Tun et al. in a study of 757 infants from the CHILD cohort found that higher frequencies of disinfectant use increased odds of a higher body mass index by the age of 3, which they determined to be mediated by the gut microbiome composition at 3-4 months (M. H. Tun et al., 2018). However, the differences in our findings may be due to the lack of a similar outcome variable, as we lack the data at 3 years to check the same mediating effect. As the cohort grows and data is collected from additional timepoints, we may have an improved ability at detecting an effect of hygiene practices, mediated through the early gut microbiome.

The limited direct contact of three-month-old infants with their environment likely also influenced the findings related to hygiene practices when compared to previous studies (Byrne et al., 2023; Sledge et al., 2022). At this age, infants are primarily carried by caregivers or placed in predetermined environments, such as cribs, which may limit their exposure to diverse microbial environments (Adolph & Franchak, 2017). Consequently, the hygiene variable may reflect the impact of hygiene practices on the more robust maternal microbiome rather than the infants' direct environmental interactions. Infants only begin to hold their head without support and sit on their own between 4-7 months, so they are limited in their individual interactions with the environment around them and the most significant exposure is likely the microbes they receive directly from their caregivers (Adolph & Franchak, 2017; Wiley et al., 2024). It is possible that

the effect played by hygiene will become more evident as the ae of the cohort increases, and as contact with the surrounding environment occurs at a higher frequency.

As discussed in the methods, our hygiene practices variable was primarily concerned with personal and household cleanliness, rather than a larger context of bacterial and infection transmission. Several studies have failed to find clear connections between household cleaning practices and large changes in the gut microbiome or related effects like the development of allergies (Robertson et al., 2023; Weber et al., 2015). Weber et al. tested the connection between a variety of household cleanliness practices and factors and the later development of allergy in the first 5 years of life. While certain dust markers protected from allergy development, the authors concluded that "neither home nor personal cleanliness impact on the development of asthma and allergies" and that "normal cleaning does not affect permanent microbial colonization of indoor environments" (Weber et al., 2015). A separate study found that exposure to triclosan-containing household and personal care products did not significantly alter the overall microbial diversity in the gut microbiotas of mothers and infants, though the sample size was fairly small (17 households in test group; (Ribado et al., 2017). These studies suggest that our measurement for hygiene practices may also have been misguided in what aspects of hygiene we chose to include. While environmental microbes are important for shaping the composition of the gut microbiome, normal household cleaning practices may have less of an impact than was previously thought and further investigation is needed to define what parts of regular 'hygiene' are impactful for the microbiome.

4.2 – Associations Between Social Contact and Hygiene with Individual Gut Microbial Taxa

To further examine the influence of social contact and hygiene practices on the infant gut microbiome, we then examined associations with microbial taxa. *Bifidobacterium* species were notably associated with social contact (Table 9). Correlation analyses revealed that several species of *Bifidobacterium*, including *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium longum*, showed significant positive associations with social contact scores ($r = 0.30$, $p = 6.25E\text{-}08$, adjusted $p = 4.25E\text{-}06$ for *B. breve*). When PCA was performed to visualize correlations between bacterial taxa and beta diversity, several *Bifidobacterium* species demonstrated a similar directional effect as social contact (Figure 4). *Bifidobacterium bifidum* and *Bifidobacterium longum* positively associated

with both PC1 and PC2, projecting towards the same quadrant (upper right) as the social contact variable (Figure 3). This alignment suggests that social interactions are positively correlated with the presence and abundance of these microbial species as well as factors represented by the principal components. Previous research has established that *Bifidobacterium* species are crucial for infant gut health due to their role in modulating the immune system and inhibiting pathogenic bacteria (Saturio et al., 2021). These bacteria are among the first colonizers of the infant gut and are highly responsive to breastfeeding and environmental influences, possibly including social interactions (Enav, Bäckhed, & Ley, 2022). *Bifidobacterium species* contribute significantly to the development of the gut microbiota, shaping its structure and function during the early stages of life. These bacteria produce short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate through fermentation processes, which play a vital role in gut health by regulating the gut barrier function, acting as energy sources for colonocytes, and modulating immune responses (Ríos-Covián et al., 2016). Studies in mice also suggest that *Bifidobacterium* species can influence the gut-brain axis, impacting neurological development and function through production of neurotransmitters and other signaling molecules (Gacias et al., 2016; Sgritta et al., 2019; Sudo et al., 2004).

While known to be associated with breastfeeding and vertically transmitted from mother to infant, there is evidence that certain species of *Bifidobacterium* can also be transmitted horizontally, from environmental sources (Hilliard & Sela, 2024). *B. longum* and *B. breve* have been noted among *Bifidobacteria* species to be specifically more tolerant to stressors like bile and oxygen, and better adapted to survive outside of the body (Andriantsoanirina, Allano, Butel, & Aires, 2013). These species, both associated with social contact in our results, have an increased chance of horizontal transfer if they can stay on surfaces for prolonged periods of time. A 2018 study by Odamaki et al. found strain transmission of *B. longum* across family members, not restricted to mother infant pairs (Odamaki et al., 2018). It is possible we are detecting a similar result in our study, but more research needs to be done to truly understand the dynamics of horizontal transfer for *Bifidobacterium* species.

However, another study by Korpela et al. (2024) of the CORAL cohort also examined social contact and the gut microbiome in the context of the pandemic with stool samples taken from infants at 6 and 12 months of age. Though they found that the development and composition of the gut microbiome occurred largely as expected, there was a reduced frequency

of spore-forming bacteria like *Clostridia*, and an increased abundance of *Bifidobacteria* in infants with reduced external exposures (Korpela et al., 2024). These results are contrary to our results, with an opposite signal of *Bifidobacteria* being negatively associated with a measure of social contact. The differences in findings could be attributed to methodological variations, such as the specific measures of social contact, the timing of sample collection (3 months versus 6 to 12 months), and the analytical approaches used. Their study separated household social contact away from exposure to humans and the environment outside of the immediate family. As our measure included household members as well as visits to other households, public places, and visits into the household, a large component was significantly different and may explain why the signal with *Bifidobacteria* was increased. Future studies that investigate social contact's impact on the microbiome in non-pandemic settings would be helpful to determine exactly which types of social interactions are most associated with specific microbial taxa.

Hygiene practices had a less pronounced effect on specific microbial taxa compared to social contact, but several were significant. *Faecalibacterium prausnitzii* and *Eubacterium rectale* showed positive correlations with hygiene scores ($r = 0.19$, $p = 0.00078$, adjusted $p = 0.046$ for *F. prausnitzii*), meaning that its presence was associated with an increase in hygiene. A possible mechanism that needs further investigation could be that a clean environment might reduce exposure to pathogenic bacteria, supporting growth of beneficial commensals such as *F. prausnitzii* (Stone, Tolmay, Tucker, & Wolfaardt, 2020). *Faecalibacterium prausnitzii* and *Eubacterium rectale* are key acetate consumers that produce butyrate, a short-chain fatty acid (SCFA) known for its beneficial effects on gut health (Louis & Flint, 2009). Butyrate is a key energy source for colonocytes and has anti-inflammatory effects. In addition to butyrate, *F. prausnitzii* produces bioactive molecules such as shikimic and salicylic acids, which further contribute to its anti-inflammatory effects. However, it is challenging to determine if *F. prausnitzii* and *E. rectale* are the primary producers of butyrate among the diverse community of SCFA producers in the gut due to functional redundancy (Louis & Flint, 2009). Additionally, the presence of specific *Faecalibacterium* species is related to other bacteria the gut microbiome increasing or decreasing (Martín et al., 2023). The exact functions of these bacteria may be less of note than what other species they associate with, and further investigation is necessary to understand the relationships specific to *F. prausnitzii* and *E. rectale*.

Aim 2: Differential expression of bacterial species across high and low social contact and hygiene practices

Our second aim was to determine microbes that were differentially present (or 'missing') in high versus low social contact and hygiene groups. To determine this, we split the sample into two groups for both primary exposure variables (high and low depending on relation to median score) and examined the proportion of samples in each group that had specific bacteria detected. Between high and low social contact, *Bifidobacterium breve* was the most differentially present species, again suggesting that social interactions promote the colonization of certain *Bifidobacterium* species in infants. For differential presence across samples, it is important to note that when a particular *Bifidobacterium* species becomes dominant, it can sometimes exclude other members of the same genus. This phenomenon, known as competitive exclusion, occurs when one species outcompetes others for resources and ecological niches (Nagao-Kitamoto et al., 2020). This competitive dynamic might explain the distinct presence patterns observed, where a high abundance of *Bifidobacterium breve* in infants with high social contact might lead to lower detection rates of other *Bifidobacterium* species, such as *B. longum*. Therefore, future research analyzing data at the genus level could provide a more comprehensive understanding of these interactions and help determine the overall impact of social contact on the gut microbiome (Yarza et al., 2014).

Hygiene practices had a less pronounced effect on the differential presence of specific microbial taxa compared to social contact, but again *Faecalibacterium prausnitzii* and *Eubacterium rectale* were the most significantly different between the two groups, both being present at a greater level in higher hygiene samples. As discussed, this finding may align with previous research indicating that certain hygienic practices can reduce pathogen load, allowing beneficial microbes to thrive (Stone et al., 2020). However, it also contrasts with some hypotheses suggesting that excessive hygiene might reduce microbial diversity and disrupt beneficial microbial communities (Wold, 1998).

Overall, our data suggests that while social contact significantly promotes the colonization of beneficial *Bifidobacterium* species, hygiene practices had a less determinable or pronounced effect on the species present in the infant microbiome. This goes against our previous hypotheses that heightened hygiene during the pandemic would reduce exposure to environmental microbes. As measured in our study, social interactions play a more measurable

and distinctive role in shaping the gut microbiome than hygiene practices, possibly due to the direct transfer of microbes through physical interactions such as touching, hugging, and sharing food or objects.

4.3 – Metabolic Pathways Associated with Social Contact, Hygiene Practices

We also performed metabolomics analysis with MetaboAnalyst to determine the metabolic pathways influenced by social contact and hygiene practices in the infant gut microbiome. Metabolomic analysis is important to utilize as many microbes influence host physiology through the metabolites that they produce or break down, and results can give a better picture of the functional activities of the microbiome (Belkaid & Hand, 2014). Evaluating concentration of different metabolites in the data with MetaboAnalyst allows for the identification of metabolic pathways and bioactive compounds that may be influenced by external factors like social contact and hygiene practices, providing deeper insights into how these factors can affect the overall health and development of infants (Vernocchi, Del Chierico, & Putignani, 2016).

Social contact was notably linked to many varied metabolic pathways, such as Alanine, Aspartate, and Glutamate Metabolism (impact score: 0.47, $p < 0.001$) and Arginine biosynthesis (impact score: 0.37, $p < 0.001$) (Table 11). These pathways play crucial roles in amino acid metabolism, which is essential for protein synthesis and neurotransmitter production (Wang et al., 2019) (Agnello et al., 2017). Social contact appears to affect a broad spectrum of metabolic processes, potentially reflecting the diverse microbial interactions facilitated by increased physical and social interactions.

In contrast, hygiene practices were associated with fewer significant metabolic pathways (Table 12). Specifically, Butanoate Metabolism (impact score: 0.11, $p = 0.001$) and Beta-Alanine Metabolism (impact score: 0.16, $p = 0.0013$) were the two pathways significantly linked to hygiene scores after correction for multiple testing. Butanoate metabolism is important to produce short-chain fatty acids (SCFAs) like butyrate, which maintain gut health and modulating the immune system (Nogal, Valdes, & Menni, 2021). The presence of significant associations as well as the limited number could suggest that hygiene practices may influence specific metabolic niches within the gut microbiome, such as SCFA production and immune modulation. This

selective influence contrasts with the broader metabolic impact associated with social contact in our results.

Additionally, for both social contact and hygiene practices, many of the identified metabolic pathways had low impact scores, indicating fewer matched metabolites or matched metabolites at non-key positions within the pathways. This can complicate the interpretation of the results, as the associations found may not fully capture the functional relevance of these pathways in the gut microbiome's metabolic network (Chong et al., 2019). As well as focusing on the detected metabolites, future research in the dataset can examine the functional potential of the gut microbiome by analyzing the genetic capacity of microbial communities to perform various metabolic functions. This could involve analyzing the genes involved in metabolic pathways that the microbiome is capable of utilizing in combination with detected metabolites, thereby offering a more comprehensive understanding of the microbial metabolic potential (Chen, Li, & Xu, 2022).

4.4 – Relationships Between the Gut Microbiome and Socioemotional Development

The Ages and Stages Questionnaires: Social-Emotional (ASQ: SE) is an essential tool for evaluating socioemotional development in infants and young children (Squires, Bricker, & Twombly, 2002). Typically, ASQ: SE scores are used to screen children across seven behavioral areas: self-regulation, compliance, adaptive functioning, autonomy, affect, social-communication, and interaction (Squires, Bricker, Twombly, et al., 2002). Normative data indicate that higher scores on the ASQ: SE reflect greater socioemotional challenges, with specific cutoff points identifying children at risk for developmental concerns (Squires, Bricker, Twombly, et al., 2002). As our study population had a higher-than-average socioeconomic study, our scores were likely positively skewed (though limited comparison datasets exist for the ASQ: SE-2 due to a low number of similar studies).

Aim 3: Determine Associations of Gut Microbiome with Socioemotional Development

The third aim of this study was to determine whether features of the gut microbiome can predict socioemotional outcomes, as measured by ASQ: SE scores. To do this, we performed the same analysis we had done in Aim 1 to identify associations between the exposure variables and microbiome features, replacing the exposure variables with the raw and cutoff scores for the ASQ: SE questionnaire.

When investigating associations with diversity, we found that raw ASQ: SE scores showed a significant positive association with Simpson alpha diversity, but cutoff scores did not demonstrate the same relationship. Higher alpha diversity, as measured by the Simpson index, was linked to worse socioemotional outcomes (higher ASQ: SE scores). Increased diversity is typically not a positive marker at the 3-month timepoint (Liu et al., 2020). At this early stage, the gut microbiome is still developing, and higher diversity might indicate instability or the presence of less beneficial microbial populations (Bäckhed et al., 2015). This may make sense with our results, as higher ASQ:SE scores indicate problems in socioemotional development, and these were associated with Simpson alpha diversity (the probability that two individuals randomly selected from a sample will belong to the same species) (Liu et al., 2020; Moroishi et al., 2022). However, the strength of this relationship is uncertain (as it was only detected by linear regression and not the more typical spearman correlation that is more flexible for non-parametric data) and requires further longitudinal studies to determine its consistency over time (Lutz et al., 2022; Thomaz F. S. Bastiaanssen, 2022).

When PCA analysis was performed, we found that the ASQ: SE scores did not exhibit a very large effect on beta diversity compared to other scores and variables (Figure 9). However, the ASQ: SE scores (raw and cut-off) exhibited an opposite directional effect on beta diversity compared to social contact. Specifically, lower ASQ: SE scores, which indicate better socioemotional outcomes, had a similar directional effect to social contact in the beta diversity analysis. This suggests that as the ASQ: SE score increases, indicating poorer socioemotional outcomes, it may have a similar but inverse relationship with the microbial community composition as had by increases in social contact. The results imply that beneficial socioemotional outcomes and increased social interactions are associated with comparable microbial community structures. It is possible that social contact may positively influence the gut microbiome in ways that are conducive to better socioemotional development, but it is equally possible that more social children have healthy socioemotional development, and the variance shift in the microbiome occurs with the development rather than causing it.

Following analysis of beta-diversity, PERMANOVA analyses were conducted to determine if socioemotional development could significantly explain microbiome variance (Table 14 – 17). When analyzed individually, ASQ: SE cutoff scores were not significant ($p = 0.054$), but the raw ASQ: SE scores were ($p = 0.035$). This could mean that the increased

variability in the raw ASQ:SE score might capture subtle variations that the cutoff scores do not, increasing its ability to explain microbiome variance across our sample. Including social contact in the model with cutoff scores improved fit and increased the statistical significance of cutoff scores, suggesting that social contact helps to better isolate and clarify the effects of ASQ: SE variables. This implies that social contact may play a crucial role in revealing the true relationship between the gut microbiome and socioemotional outcomes. Given that social contact is itself a presumed major influence on socioemotional development, this finding may indicate that accounting for social contact variability allows for a more accurate assessment of how microbial composition is associated with socioemotional behavior. Interestingly, the significance of the raw ASQ: SE scores was not notably affected by the inclusion of social contact. This could mean that while raw scores provide a broader measure of socioemotional outcomes, the cutoff scores, when paired with social contact, highlight specific group differences more effectively. However, it's important to note that the bivariate relationship between social contact and ASQ: SE scores was not significant in our study. This implies that, while social contact theoretically influences socioemotional behavior, our data did not show a direct significant association. The role of social contact appears to be complex; it may not directly influence ASQ: SE scores in a bivariate analysis, but it could still play a role in the relationship between the microbiome and socioemotional outcomes.

Taxa associations were also investigated in relation to ASQ: SE scores (Table 18 and 19). *Streptococcus salivarius* was significantly associated with lower raw ASQ: SE scores, suggesting a potential beneficial role in social-emotional development. Identified in the literature as a potential probiotic strain, *S. salivarius* has been shown to protect against infections and support immune homeostasis (MacDonald et al., 2021). One of the first colonizers of both the oral and gut microbiome in infants, *S. salivarius* is thought to reduce inflammation through inhibiting activation of the NF-κB pathway (Kaci et al., 2014). The connection between the gut microbiome and the immune system is one pathway through which the gut microbiome plays a critical role in brain development and function by regulating neuroinflammation, synaptic pruning, and neurogenesis (Li & Barres, 2018). The gut microbiome can impact brain development and function through the immune system and other independent pathways of brain axis communication, modulating neural circuits and behaviour via various signalling pathways, such as the vagus nerve (Sherwin et al., 2018).

There is additional evidence in animal models that supports that the types of bacteria present, rather than just their diversity, might be critical for socioemotional outcomes. As mentioned in the introduction, a study by Sgritta et al. (2019) found that ASD-like symptoms in maternal high-fat diet (MHFD) mice offspring were linked to alterations in the gut microbiome, specifically a reduction in *Lactobacillus reuteri*, but administering *L. reuteri* improved social behavior through an increase in production of oxytocin and activation of the vagus nerve, demonstrating the potential of specific bacterial strains to influence sociability in mouse models (Sgritta et al., 2019). These results suggest that a single bacteria can have dramatic effects on social behaviour in mice. Further research with additional timepoints should be conducted to determine the long-term strength of this association and relationship between *S. salivarius* and socioemotional development, as well as immune markers that may be associated.

Few metabolic pathways were associated with ASQ:SE scores when analyzed with MetaboAnalyst (Table 20 and 21). However, the Ascorbate and Aldarate Metabolism pathway was significantly associated with both raw and cutoff ASQ: SE scores, suggesting a possible role in socioemotional development. The gut microbiome can influence physiological processes such as brain development and function by producing and metabolizing different molecules and metabolites (Hsiao et al., 2013). For the raw scores, the Ascorbate and Aldarate pathway showed a significant p-value ($p = 0.037$) even after correction for multiple testing (FDR). Despite the statistical significance, the impact score of 0 suggests that the overall pathway alteration might be minimal in the context of socioemotional development (Pang et al., 2022).

Aim 4: Gut Microbiome Mediation of Socioemotional Development Through Beta Diversity

The fourth aim sought to investigate whether the gut microbiome mediates the relationship between social contact and hygiene habits and socioemotional development. Both social contact and hygiene scores did not show significant direct correlations with ASQ: SE scores, and linear regression analyses confirmed the absence of significant relationships between these variables. Despite the absence of any direct effect, mediation analysis was pursued as it had been a part of the original research plan. Due to the similar directional effect or low ASQ:SE scores and high social contact scores, we investigated PC1 and PC2 as mediators (Figure 11). The mediation results provided evidence suggesting an indirect effect, where the gut microbiome, represented by PC1 from the PCA of beta diversity, partially mediated the relationship between social contact and socioemotional outcomes. Specifically, the Average

Causal Mediation Effect (ACME) just met the threshold value of significance (p =0.044). This could indicate that there is a mediating role of the gut microbiome in the model, but the lack of strong results necessitates further research with more timepoints and a larger sample size to truly understand the relationship. Examining the microbiome at multiple time points throughout the first year of life could offer a clearer picture of how early microbial changes correlate with socioemotional development over time (Lyu, Qu, Divaris, & Wu, 2024). The gut microbiome is still developing at three months of age and may not yet be stable enough to predict socioemotional outcomes at one year (Bäckhed et al., 2015). Additionally, incorporating more diverse populations and controlling for confounding variables such as diet and antibiotic use will enhance the robustness and generalizability of the findings.

4.5 – Strengths and Limitations of the COVID-19 Pandemic

The COVID-19 pandemic provided a unique opportunity to analyze the impact of hygiene and social contact on the microbiome. With many families adhering to similar lockdown measures and social distancing guidelines, there was a relative uniformity in external conditions. The heightened awareness and practices of hygiene, coupled with limited social interactions, created a distinctive environment to examine the 'Old Friends' hypothesis in real-time. However, these same conditions also posed several challenges. Lockdowns and social distancing measures meant that many children had limited opportunities for physical interaction with peers, which is a crucial component of socioemotional development (Ebrahimi et al., 2022; Finlay et al., 2021). Additionally, increased stress levels among parents, due to health concerns and economic uncertainties, could have further affected the socioemotional climate at home. This elevated stress might translate to altered parent-child interactions, potentially impacting the children's development and their ASQ: SE scores (Masarik & Conger, 2017; Provenzi et al., 2023). Given these conditions, the pandemic serves as a potential confounding factor in our results.

Specific to our sample and the context of the COVID-19 pandemic, another limitation may have been the habits of our sample itself. The majority of the mothers in our sample indicated through survey responses that they were following pandemic guidelines (i.e., getting vaccinated and limiting gatherings) and that they cleaned regularly in their homes. We may not have had sufficient variability in hygiene habits and attitudes to detect more subtle effects. A significant proportion of the sample also had similar backgrounds, being majority Caucasian and

middle class (Table 1), and primarily from urban environments. A more diverse sample may have been needed to detect effects that arise from variations in cleaning practices or attitudes to the pandemic that may reflect in hygiene practices (Lee et al., 2021; Sultana et al., 2022). Additionally, variations in social interactions and increased parental stress during the pandemic could have affected children's socioemotional development, potentially leading to atypical ASQ: SE scores (Duguay et al., 2022; Provenzi et al., 2023). Studies show that stress in caregivers has been shown to influence their interactions with children and the overall emotional climate of the household, which can affect children's socioemotional well-being (Masarik & Conger, 2017). This makes it difficult to disentangle the effects of the gut microbiome from the broader impacts of the pandemic on socioemotional development. In future research it may be beneficial to examine differences in microbiome samples collected during low and high lockdown periods or use measures of restriction stringency as a variable in analyses to see if there were measurable effects of pandemic lockdowns and behaviours.

4.6 – Future Directions

The role of social contact appears robust, but the influence of hygiene practices requires further clarification, particularly as infants grow older and their interactions with their environment increase. To further understand the complex interactions between the gut microbiome, social contact, hygiene practices, and socioemotional development, several avenues could be explored. Most impactful would be to examine data at additional timepoints, especially in connection with socioemotional development (Lyu et al., 2024). This would be valuable to determine if the relationships observed in this study become more pronounced later in development. For instance, hygiene practices might have a more significant impact on the gut microbiome at six months or one year of age compared to the three-month timepoint examined in this study. Investigating relationships with socioemotional scores could clarify if socioemotional outcomes are better predicted by microbiome features at corresponding developmental stages.

Additionally, it would be beneficial to investigate the influence of other variables more deeply. For example, breastfeeding or other factors (investigated as covariates in this study) could potentially act as a protective factor and preserve the presence of *Bifidobacterium* species even in infants with low social contact. Understanding how these factors interact with social and environmental variables will provide a more comprehensive view of the factors that support a

healthy gut microbiome. Other factors, investigated as covariates in this study, may be worth investigating more closely in combination with our scores, to determine if there are any between-group factors that may have significant impacts on the microbiome in relation to the exposures.

In future cohorts, expanding studies to include more diverse populations and detailed behavioral assessments can help capture a broader impact of microbial changes on behavior and development. Including participants from various socio-economic, ethnic, and geographic backgrounds will ensure that the findings are generalizable and applicable to a wider population. This diversity can also reveal unique interactions between the gut microbiome and environmental factors specific to different places and groups, shedding light on population-specific influences.

4.7 – Summary and Conclusions

This study provides evidence that social contact and hygiene practices are associated with features of the infant gut microbiome. However, the impact of these microbiome changes on socioemotional development at one year of age, while measurable, appear to be small and require further investigation and validation.

Social contact has significant associations with the gut microbiome, with specific taxa showing strong correlations with social contact scores. Notably, *Bifidobacterium* species, particularly *Bifidobacterium breve*, are closely associated with higher levels of social contact. These findings suggest that social interactions play a crucial role in shaping the microbial composition of the infant gut. In contrast, hygiene practices have a less pronounced impact on the gut microbiome compared to social contact. Although fewer significant microbial associations were observed, hygiene practices still exert a measurable effect on the microbial community, indicating that while hygiene is an important factor, its influence on the gut microbiome is more subtle.

Specific features of the gut microbiome, including alpha diversity and certain taxa, correlated with socioemotional outcomes. The gut microbiome, as represented by the first principal component (PC1) in beta diversity analysis, may mediate the effect of social contact on ASQ: SE scores, though this effect is not strongly significant. Despite the observed variations in gut microbiome composition associated with social contact and hygiene practices, there was no strong evidence linking these microbial changes directly to the socioemotional outcomes measured by ASQ: SE scores.

Our findings suggest that having a greater number of social contacts in early life can positively influence the gut microbiome, promoting the presence of beneficial taxa often linked to better infant health. There may be potential benefits of fostering rich social environments for young infants, not only for their immediate social and emotional wellbeing but also for their long-term health through a well-developed microbiome.

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