THE MICROBIOTA GUT BRAIN AXIS

The development of the gut microbiota and mental health over the first 14 years of life

Yangwenshan Ou

Propositions

1. Complete maturation of the gut microbiota extends from infancy into childhood and puberty.
   (this thesis)

2. The gut microbiota is a regulator of child mental health and development.
   (this thesis)

3. A single model never represents all aspects of an actual condition.

4. Omics-driven correlative results are often overinterpreted without adequate evidence from mechanistic validation studies.

5. Unconsciously under-reporting negative results introduces bias when drawing conclusions.

6. What we see is often obscured, and we always want to know what is blocked from our view.

7. It does not matter what we choose, but the process of selection does.

Propositions belonging to the thesis, entitled

The microbiota-gut-brain axis: the development of the gut microbiota and mental health over the first 14 years of life

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The microbiota-gut-brain axis:

the development of the gut microbiota and mental health over the first 14 years of life

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Chapter 1

General introduction and thesis outline

Brief introduction

There is mounting evidence that the gut microbiota is intimately involved in a wide variety of health outcomes, from energy supplementation to immune regulation. Remarkably, recent investigations, especially animal studies and human case-control studies, have illuminated the emerging role of the gut microbiota in mental health. Therefore, it is essential to keep normal microbial composition and function to maintain both physical and mental fitness. As a consequence of multiple early-life factors, the gut microbiota shows a dynamic development in infancy and toddlerhood, and is supposed to strongly resemble an adult microbial profile within the first three years of life. However, due to the scarcity of long-term longitudinal studies, it is not yet clear whether microbial community succession continues to an older age under the extended influence of extrinsic factors. Besides, little is known about how rapidly developing child gut microbiota is related to mental wellbeing during childhood and later in life in community samples during sensitive periods.

To provide insight into the unclear and the unknown, this thesis aims to (1) describe gut microbiota development and its associations with extrinsic factors from birth to puberty and (2) explore relations between the gut microbiota and child mental development and health in community samples during sensitive time windows. In the following sections, I will take you on an explorative journey to gut microbiota development and microbial relations to multiple extrinsic factors and health outcomes, in particular child mental wellbeing.

What is the gut microbiota?

The term microbiota is defined as the assemblage of microorganisms present in a certain environment (Marchesi & Ravel, 2015), consisting of two ancient Greek words "micro" indicating small and "biota" specifying organisms in an ecosystem or a particular area (Berg et al., 2020). Microorganisms include the aggregate of bacteria, archaea, and eukaryotes, of which those specifically inhabiting the gastrointestinal system are collectively termed the gut microbiota. Gut bacteria, approximately accounting for 10^{14} cells per person (Sender, Fuchs, & Milo, 2016), have been widely explored in many aspects of health and disease (Fan & Pedersen, 2021; Nikolova et al., 2021), since 1885 when they were first studied in infants by Professor Theodor Escherich (Farré-Maduell & Casals-Pascual, 2019). Recently, with increasing knowledge on the microbiota and its complex interplay with the host, scientists often refer to the microbiota with a broader term, "microbiome", which not only contains the community of microorganisms, but also their structural elements (e.g., proteins, lipids, polysaccharides, and nucleic acids), metabolites (e.g., organic and inorganic compounds, signaling molecules, and toxins), and environmental conditions (Berg et al., 2020). The community of microorganisms not only provides the host with nutrients and energy, but also regulates bowel movements, immune response, and even mental health (Cryan et al., 2019; Flint, Scott, Louis, & Duncan, 2012; Valdes, Walter, Segal, & Spector, 2018) For easy understanding, the more universal term gut microbiota will be used to represent gut bacteria herein.

Factors related to the gut microbiota in the first years of life

It remains a general dogma that the fetus is sterile in healthy women, as a consequence of the placental barrier that protects against the invasion of pathogens and the diffusion of toxins (Matamoros, Gras-Leguen, Le Vacon, Potel, & de La Cochetiere, 2013; Rodríguez et al., 2015). However, this dogma has been challenged over the past decade, as evidence is growing that microbial DNA has been detected in meconium and amniotic fluid (Stinson, Boyce, Payne, & Keelan, 2019). Nevertheless, there is currently no consensus on whether the microbiota colonizes the fetal gut prior to birth, as both meconium and amniotic fluid are low in microbial biomass, and therefore can be easily contaminated by external materials and environment (Stinson et al., 2019).

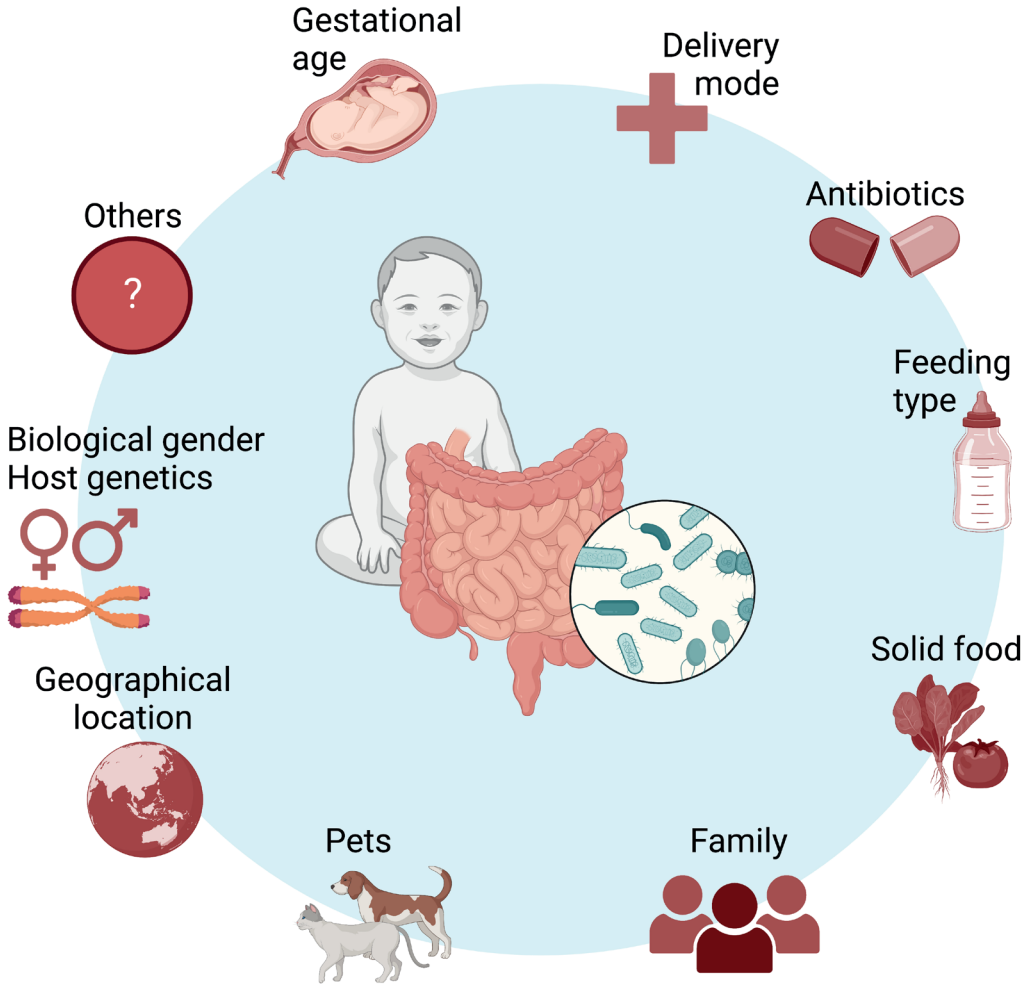

Figure 1. Factors related to the gut microbiota in the first years of life. (created with https://biorender.com/)

Despite this prenatal contention, upon delivery, the neonatal gut starts to harbor a large number and a wide array of microbes (Bäckhed et al., 2015; Hill et al., 2017; Shao et al., 2019), the composition of which is related to multiple factors (Figure 1). Vaginally delivered newborns acquire *Bifidobacterium* spp. from their mothers (Shao et al., 2019), while infants born by C-section miss the maternal transmission of beneficial microbes, which can disrupt normal gut microbiota development and further affect the immune system extendedly (Korpela, 2021). Antibiotic administration and early gestation (i.e., premature infants born before 37 weeks) can also largely impact the colonization of the gut by microbial communities at birth (Rodríguez et al., 2015). Furthermore, the feeding type exerts a considerable selection on neonatal gut microbiota. Specifically, human milk is enriched in oligosaccharides that promote the growth of benign *Bifidobacterium* spp. which can boost host immunity and protect against pathogens (Laursen, 2021). In comparison with breast-fed infants, formula-fed infants usually exhibit higher prevalence of opportunistic pathogens and a fermentation pattern towards proteolysis (Laursen, 2021). Later, complementary solid foods induce a remarkable shift in gut microbiota composition. Especially microbial taxa consuming dietary fibers will prosper, making the infant metabolically ready for an adult diet (Laursen, 2021). Taken together, feeding-related factors, such as duration of exclusive breastfeeding, proportion of breast milk to total milk intake, and the introduction of solid foods, are considered important factors affecting gut microbiota colonization and development in the first few years of life, while their long-term effects are ambiguous. Extrinsic factors related to the living environment, such as household composition (e.g., siblings and pets) and geographical location, are also linked to microbial development and composition (Gacesa et al., 2022).

Additionally, factors of hosts themselves, such as biological gender and host genetics, may also impact the structure and function of the gut microbiota (Korpela et al., 2021; Rodríguez et al., 2015; Yuan, Chen, Zhang, Lin, & Yang, 2020). Regarding biological gender, its role in infancy and middle childhood remains unclear, but differential microbial composition has been observed in children at the onset of puberty, with sexual hormones probably being the driving force (Korpela et al., 2021; Yuan et al., 2020). As for host genetics, although their interactions with the gut microbiota are still under debate at population level, several twin studies have shown profound effects of host genotype on shaping microbial communities (Rodríguez et al., 2015).

Development of the gut microbiota at early ages

Under the joint effects of the aforementioned factors, the gut microbiota may follow divergent trajectories to develop into different microbial patterns, varying in composition and possibly function. It has been believed that the gut microbiota completes developing to an adult-like configuration within the first three years of life (Yatsunenko et al., 2012), while some controlled studies (children vs adults from different cohorts) have indicated that it may take longer to reach a mature state highly resembling adult gut microbial profiles than previously thought (Agans et al., 2011; Cheng et al., 2016; Hollister et al., 2015; Ringel-Kulka et al., 2013). According to these cross-sectional studies, one of the major differences between

older children and adults is the relative abundance of *Bifidobacterium* spp., which were reported conspicuously higher in infants and children. However, evidence from longitudinal studies that include continuous time points from the same cohort, is scarce beyond the age of three years.

The gut microbiota in health and diseases

Over the years, many health outcomes and diseases have been found tightly related to the gut microbiota (de Vos, Tilg, Hul, & Cani, 2022). In healthy individuals, the gut microbiota mainly supplies nutrients, provides energy, inhibits growth of pathogens, maintains normal gut motility and immune function (Flint et al., 2012; Valdes et al., 2018). When a disturbance happens to the gut microbiota, these microbial functions can be influenced partly or completely, which may contribute to the pathogenesis of various diseases or disorders, such as gastrointestinal diseases, metabolic disorders, and neuropsychiatric and neurogenerative illness (Cryan et al., 2019; Fan & Pedersen, 2021; Flint et al., 2012; Fujimura & Lynch, 2015; Meng, Bai, Brown, Hood, & Tian, 2018; Nishida et al., 2018; Pittayanon et al., 2019). In particular when the microbial disruption occurs during sensitive time windows, like infancy and puberty, this may not only influence host fitness temporarily, but also long-term and even life-long (Derrien, Alvarez, & de Vos, 2019).

The microbiota-gut-brain axis

In the past decades, scientists found that germ-free, antibiotic-treated, or gnotobiotic rodents, showed substantial changes in brain physiology and host behavior (Cryan et al., 2019). These findings first jointly established the emerging role of the gut microbiota in brain development and mental health. In the following years, microbial differences were frequently observed between neurotypical individuals and patients with various mental illnesses, such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), major depressive disorder (MDD), and generalized anxiety disorder (GAD) (Bundgaard-Nielsen et al., 2020; Cheung et al., 2019; Jiang et al., 2018; Sukmajaya, Lusida, Soetjipto, & Setiawati, 2021). Meanwhile, animal studies were carried out to discover potential molecular mechanisms underlying the observations. Collectively, these links between the gut and the brain are defined as the microbiota-gut-brain axis (MGBA) (Cryan et al., 2019; Morais, Schreiber, & Mazmanian, 2021). Along this MGBA, three major bidirectional communication paths have been identified till now, including metabolic and endocrine pathways, immune and neuroimmune pathways, and neuronal signaling (Cryan et al., 2019; Morais et al., 2021). Importantly, it has been suggested that the interplay along the MGBA is much more active at a developing age (Cryan et al., 2019).

Across the lifespan, the period from birth to puberty is of high importance (Blakemore, Burnett, & Dahl, 2010; Tierney & Nelson, 2009). In infancy and early childhood, children experience rapid physical, emotional, and particularly cognitive developments as a result of remarkable anatomical changes in the brain (e.g., neuronal cell proliferation, migration, myelination, and synaptogenesis) (Tierney & Nelson, 2009). Of note, the development of cognition (i.e., activities and processes including thinking, reasoning,

perceiving, imaging, and remembering) in early life has large impacts on physiological and mental health in later life (Bayne et al., 2019). For instance, lower cognitive ability at early ages is associated with a higher risk of alcohol abuse, MDD, and GAD in adulthood (Gale et al., 2008).

In childhood and puberty, a vast repertoire of problem behavior can start emerging (Bongers, Koot, van der Ende, & Verhulst, 2003). For ease of interpretation, problem behavior is often divided into internalizing and externalizing behavior (Achenbach, 1966). Internalizing behavior includes withdrawal, anxiety, and emotional problems, mainly influencing the internal psychological environment. Externalizing behavior comprises impulsive, aggressive, and hyperactive features, which mainly exhibit in the external environment. Children with elevated levels of problem behavior are more likely to develop substance abuse, antisocial personality disorder, and depression in adulthood (McGue & Iacono, 2005).

Such an important period is referred to a sensitive time window for mental development. Under the influence of external factors during a sensitive period, it may be difficult without tremendous efforts to redirect mental development along a typical trajectory (Meredith, 2015; Nelson, Zeanah, & Fox, 2019). Remarkably, the sensitive periods of mental development seem in parallel with the sensitive periods of gut microbiota development (Cryan et al., 2019), indicating probably stronger interplay between them during such periods. However, to date, many aspects concerning the MGBA are not yet clear in community samples during sensitive time windows. In Table 1, I present currently available findings regarding the relations between the gut microbiota and cognition and problem behavior in a community sample of children.

Table 1. Findings of the relations between the gut microbiota and cognition and problem behavior in a community sample of children.

Parameter	**Design**	**Sample size; Ages; Country**	**Measures***	**Main findings**	**Reference**
Cognition	Longitudinal	N = 89; Ages = one and two years; USA	Fecal microbiota composition at the age of one year was analyzed by 16S. Child cognition was measured at one and two years of age, by the MSEL and sMRI.	Highest cognitive scores at age two were observed in the bacterial cluster enriched in *Bacteroides* at age one, while lowest scores at age two were found in the cluster enriched in *Faecalibacterium* at age one. Alpha diversity (Chao1, observed species, and Shannon index) at age one was negatively	(Carlson et al., 2018)

			related to cognitive scores at age two, and positively linked to regional gray matter volumes at age two.	
Longitudinal	N = 309; Ages = three to six months and three years; USA	Fecal microbiota composition of children aged from three to six months was analyzed by 16S. Parents completed the ASQ when children were three years old.	The *Clostridiales*-predominated coabundance group was related to worse cognitive performances in aspects of communication skills and personal and social skills. The *Bacteroides*-predominated group was related to poorer ability in fine motor skills.	(Sordillo et al., 2019)
Cross-sectional	N = 39; Age = one year; USA	At one year of age, fecal microbiota profiles were analyzed by 16S, and brain functional connectivity was measured by fMRI.	Alpha diversity was negatively related to functional connectivity regarding cognitive development.	(Gao et al., 2019)
Cross-sectional	N = 46; Age = three years; China	At three years of age, fecal microbiota configuration was analyzed by 16S. Child neurodevelopment was measured by parental BSID (second edition).	The coabundance group enriched in *Faecalibacterium*, *Sutterella*, and *Clostridium* cluster XIVa, exhibited positive relations to better mental and psychomotor developments at age three.	(Rothenberg et al., 2021)
Cross-sectional	N = 71; Age = 18 months; Spain	Fecal microbiota composition was analyzed by 16S. Child neurodevelopment was measured by the BSID (third edition) at 18 months of age.	Fine motor skills explained partial microbial variation (beta diversity). The bacterial cluster enriched in *Firmicutes* genera showed higher scores in fine motor skills than those in *Bacteroidetes* predominant cluster. Increased *Bifidobacterium* and *Lactobacillus* were related to more fine	(Acuña et al., 2021)

				motor skills, while *Turicibacter* and *Parabacteroides* were related to less fine motor skills.	
	Longitudinal	N = 260; Ages= six weeks and one to three years; USA	Fecal microbiota diversity and genus-level composition were analyzed at the age of six weeks, one year, and two years, by 16S. Fecal microbiota composition at species-level was analyzed at the age of six weeks and one year by WGS. Parents completed the BASC (second edition) when children were three years old.	Regarding 16S results, increased relative abundances of *Bifidobacterium*, *Bacteroides*, and *Streptococcus* at six weeks were related to more adaptive skills in boys, while increased levels of *Klebsiella*, *Clostridium*, and *Haemophilus* at the same age were related to less adaptive skills in boys. Regarding metagenomic outcomes, increased relative abundances of *Klebsiella oxytoca* were related less adaptive skills in boys.	(Laue et al., 2021)
	Longitudinal	N = 405; Ages = one and two years; Canada	Fecal microbiota profiles were analyzed at the age of one year by 16S. Child neurodevelopment was measured by parental BSID (third edition) at one and two years of age.	The bacterial cluster enriched in *Proteobacteria* at age one exhibited lowest cognitive scores at age two, compared to *Firmicutes* and *Bacteroidetes* predominant clusters.	(Tamana et al., 2021)
	Longitudinal	N = 90; Ages = birth to 60 months; Italy	Microbiota composition was analyzed in meconium and fecal samples at the age of three, six, 12, 24, and 36 months by 16S. Cognitive development was measured at the age from six to 60 months by the GMDS.	Gut microbiota composition in meconium was associated with practical reasoning at 60 months of age. In particular, increased relative abundances of *Bifidobacterium* were related to better practical reasoning. (The authors did not report links between	(Guzzardi et al., 2022)

				the gut microbiota and cognition at other ages. This might be their next step.)	
Problem behavior	Longitudinal	N = 201; Ages = one month to two years; Australia	Fecal microbiota composition was analyzed at the age of one, six, 12 months, by 16S. Parents filled in the CBCL when children were two years old.	Increased relative abundances of *Prevotella* at 12 months of age were related to less problem behavior at the age of two years. Increased relative abundances of an unidentified *Lachnospiraceae* genera at the age of 12 months were related to more problem behavior at age two. Both associations were primarily related to internalizing behavior.	(Loughman et al., 2020)
	Longitudinal	N = 260; Ages = six weeks to three years; USA	Fecal microbiota diversity and genus-level configuration were analyzed at the age of six weeks, one year, and two years by 16S. Species-level fecal microbiota composition was analyzed at the age of six weeks and one year by WGS. Parents completed the BASC (second edition) when children were three years old.	Increased levels of alpha diversity (Shannon index from 16S) at the age of six weeks were related to less internalizing behavior at age three, particularly in boys. Increased relative abundances of *Streptococcus peroris* at age one were related to less internalizing behavior at age three only in girls.	(Laue et al., 2021)
	Cross-sectional	N = 248; Age = four years on average; Canada	Fecal microbiota composition was analyzed at the age ranging from three to five years by 16S. Fecal short-chain fatty acid levels were measured by LCMS. Parents completed the CBCL for children.	Increased Shannon alpha diversity was related to less internalizing behavior. Increased levels of valerate and isobutyrate were related to less internalizing behavior.	(Van De Wouw et al., 2022)

*Notes. 16S, 16S ribosomal RNA gene sequencing; WGS, whole-genome shotgun metagenomic sequencing; MSEL, the Mullen Scales of Early Learning; sMRI, structural magnetic resonance imaging; ASQ, Ages and Stages Questionnaire; fMRI, functional magnetic resonance imaging; BSID, Bayley Scales of Infant Development; BASC, Behavioral Assessment System for Children; GMDS, Griffith's Mental Development Scales; CBCL, Child Behavior Checklist; LCMS, liquid chromatography–mass spectrometry.

Approaches to decipher gut microbiota composition

Given the fact that a large number of microorganisms cannot grow on known laboratory media (Eckburg, 2005; E. J. Stewart, 2012), culture-independent molecular approaches have been developed rapidly in the past decades. These approaches decipher gut microbiota composition by classifying DNA sequences taxonomically and phylogenetically (Poretsky, Rodriguez-R, Luo, Tsementzi, & Konstantinidis, 2014). This process allows quick, accurate, and comprehensive descriptions of microbial community structure and dynamics. Such DNA-based strategies can be categorized into targeted amplicon sequencing and whole-genome shotgun metagenomic sequencing (Kuczynski et al., 2012). The former focuses on PCR amplification of marker genes, while the latter sequences random fragments of genome without targeting and amplifying a specific gene (Kuczynski et al., 2012). Regarding the amplicon-based approach, 16S ribosomal RNA (rRNA) genes have been commonly used as marker genes for prokaryotes, as these genes not only contain highly conserved regions that can be targeted by the universal PCR primers but also hypervariable regions that can be distinguished between microbial taxa (Kuczynski et al., 2012). To date, many gut microbiota studies are based on 16S rRNA gene amplicon sequencing, although they can largely differ in sample collection, DNA extraction, PCR primers, sequencing platforms, and bioinformatic pipelines, which might cause inherent biases between study comparisons. Furthermore, compared to shotgun metagenomic sequencing, 16S rRNA gene sequencing is restricted by the targeted region and primer specific amplification bias, lower microbial resolution, and reduced accuracy in predicting gene functions (Brumfield, Huq, Colwell, Olds, & Leddy, 2020; Durazzi et al., 2021; Ramiro-Garcia et al., 2018). Despite these limitations, 16S rRNA gene sequencing provides affordable and informative data about microbial communities, and therefore is irreplaceable at this moment.

Disentangling relations between gut microbiota composition and host observable traits

Targeted amplicon sequencing and whole-genome shotgun metagenomic sequencing generate numerous gut microbiota data, which are often (1) high-dimensional, i.e., the number of microbial taxa is larger than the number of samples, (2) phylogenetically structured, i.e., evolutionary closeness between microbial taxa, (3) zero-inflated, i.e., some microbial taxa are absent in samples, and (4) over-dispersed, meaning that variance exceeds the mean (Chen & Chen, 2018). These features make it a challenging task to explore sequence-based gut microbiota composition in relation to host observable traits (e.g., age, BMI, biological gender, and mental health).

Depending on the type of analytical strategies, current methods can be divided into classic and modern tools:

Classic univariate tools, such as parametric t-test and non-parametric Wilcoxon rank sum test are often used to compare microbial differences in abundances and diversity between two groups. Comparisons between more than two groups can be done by using their expansions, i.e., ANOVA and Kruskal-Wallis test. Despite the fact that these tools are easy-to-perform, such basic tools do not consider the existence of confounders. In contrast, by

performing a more flexible model, such as a generalized or Bayesian linear model used in this thesis, we can contemplate potential confounders, and therefore reduce the correlational bias. For instance, Valles-Colomer et al. fitted a generalized linear model between a mental index and abundances of a microbial taxon, along with the correction for anthropometric and gastrointestinal variables (Valles-Colomer et al., 2019). Furthermore, Eckermann et al. regressed executive functions to microbial diversity through Bayesian linear models, by taking age, gender, and socioeconomic status as potential confounders into account (Eckermann, Ou, Lahti, & de Weerth, 2022).

Classic multivariate analyses for gut microbiota studies mainly come from ecological and environmental science (Chen & Chen, 2018). Among them, constrained methods, including canonical correspondence analysis (CCA) and redundancy analysis (RDA), have been used the most frequently. In particular, RDA accepts the use of a distance or (dis)similarity matrix, which can be calculated by various measures for different purposes. For example, UniFrac distance considers phylogenetic relations between microbial taxa (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011). In particular, unweighted and weighted UniFrac distances are widely adopted in gut microbiota studies. The former assigns more weight to rare taxa, while the latter underlines the most enriched taxa. In addition to RDA, I briefly introduce two more recently developed multivariate analysis approaches (i.e., random forest and Dirichlet multinomial mixtures; both were utilized in this thesis) below.

The random forest (RF) algorithm is non-parametric. Stanislawski et al. used RF models to decipher associations of microbial abundances and diversity in the first two years of life with BMI at the age of 12 years (Stanislawski et al., 2018). They further selected important microbial features from the RF models, and then fitted a linear model to each feature, for the purpose of correlational direction and strength. Besides, RF models were adopted in predicting chronological age by the gut microbiota (Gasparrini et al., 2019; Huang et al., 2020; Wan et al., 2022). Gasparrini et al. used such a predictive tool to explore if preterm gut microbiota would develop into an age-matched healthy profile in the first years of life (Gasparrini et al., 2019). With such a tool, Wan et al. observed lagging gut microbiota development in autistic children compared to age-comparable neurotypical peers (Wan et al., 2022). As for Vujkovic-Cvijin et al., they employed RF models multiple times on numerous host observable traits about physiology, lifestyle, and diet, and assessed their individual relations to gut microbiota composition (Vujkovic-Cvijin et al., 2020).

In 2012, Holmes et al. developed a powerful framework, called the Dirichlet multinomial mixtures (DMM), which can be used to identify compositional patterns. Instead of focusing on a distance or (dis)similarity matrix as RDA does, the DMM approach pays attention to actual taxon abundances. To the best of my knowledge, the DMM has been used in describing maternal gut microbiota during pregnancy (Berry et al., 2021) and temporary gut microbiota development in early life (Roswall et al., 2021; C. J. Stewart et al., 2018), and classifying enterotypes in the gut microbial community landscape (Brial et al., 2021; Costea et al., 2017). These DMM clusters, which differ in microbial composition, can then be compared in aspects of a collection of host observable traits.

Research aims and thesis outline

Maintaining a normal developmental trajectory of the gut microbiota is pivotal for child growth and health. A derailment of the development of the gut microbiota may trigger various temporary and enduring diseases and disorders. Studying gut microbiota development during sensitive time windows can help increase the understanding of the dynamics of microbial ecology, weigh the impact of extrinsic factors, and provide leads towards effective interventions. Moreover, the sensitive time windows of the developing gut microbiota largely align with those of the maturing brain. During these periods, intense and complex bidirectional interactions between the microbiota and the brain may occur along the MGBA. Knowing how the gut microbiota is related to mental development and health is the first and primary step before systematically exploring cause and effect.

The aims of this thesis are: (1) Describing gut microbiota development and its associations with extrinsic factors from birth to puberty; (2) Exploring relations between the gut microbiota and child mental development and health in community samples during sensitive time windows. The thesis includes six chapters:

Chapter 1 introduces the importance, extrinsic factors, and analytical methods of investigating gut microbiota development, and presents the MGBA by highlighting currently available correlational findings about child cognition and problem behavior during sensitive periods.

Chapter 2 describes the associations between the gut microbiota in the first three years of life and child problem behavior and cognitive ability regarding executive functions at age three, in a longitudinal study named BINGO.

Chapter 3 provides insights into gut microbiota development in the first decade of life and its short- and long-term relations to potential extrinsic factors. The chapter also investigates microbial links to problem behavior at six and ten years of age by using several complementary and sophisticated statistical approaches, based on an ongoing longitudinal study called BIBO.

Chapter 4 depicts biological gender differences in gut microbiota composition of BIBO participants at the onset of puberty (age 12) and investigates cross-sectional associations of the gut microbiota (relative and absolute abundances of microbial taxa, and microbiota-derived fecal metabolites) with problem behavior and prosocial behavior at this age.

Chapter 5 extends the data and analyses presented in **Chapter 3** by profiling gut microbiota development in BIBO children during puberty, and delineating relations between pubertal gut microbiota and problem behavior and social anxiety from birth to puberty (age 14).

Chapter 6 discusses major findings in this thesis and proposes perspectives for future studies.

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Chapter 2

A longitudinal study of the gut microbiota during the first three years of life: links with problem behavior and executive functions in toddlerhood

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Abstract

Background: Recent studies show that the gut microbiota is critically linked to mental health. As early life is a sensitive period for both microbiota and brain development, evidence from longitudinal studies on low-risk populations are necessary. However, studies in this period are lacking. This study investigated the associations of the gut microbiota in the first three years of life with problem behavior and executive functions in three-year-old children.

Methods: Participants were low-risk three-year-old children and their parents (n=64). Fecal microbiota composition was analyzed at five different time points within the first three years of life by 16S ribosomal RNA gene sequencing. Microbial relations to three-year-old children's problem behavior and executive functions (reported by parents), and inhibition (measured by behavioral tasks), were determined by random forest and Bayesian models.

Results: Increased *Streptococcus* relative abundance, specifically at the age of two weeks and throughout the first three years of life, was related to worse executive functions. Higher relative abundance of [*Ruminococcus*] *torques* group, at the age of three years and over the period from the age of one to three years, was associated with less internalizing behavior. Besides, several robust age-specific associations were identified: higher *Bifidobacterium* relative abundance (age three years) was associated with more internalizing and externalizing issues; higher *Blautia* (age three years) relative abundance was linked to less internalizing behavior; and increased relative abundance of an unidentified *Enterobacteriaceae* genus (age two weeks) was related to more externalizing behavior.

Conclusions: Our findings provide suggestive evidence for associations between gut microbiota variation, during the first three years of life, and problem behavior, executive functions, and inhibition in a low-risk sample, supporting the idea that early life gut microbiota markers can be linked to behavioral and cognitive development.

Keywords: Gut microbiota; Early life; Problem behavior; Cognition; Executive functions; Inhibitory control

Introduction

The human gut harbors a great number of microorganisms, of which bacteria are an essential part. These microorganisms are collectively termed the 'gut microbiota' (Thursby & Juge, 2017). Not only has the gut microbiota been involved in many health outcomes, such as obesity, type 2 diabetes, and irritable bowel syndrome (Vos, Tilg, Hul, & Cani, 2022), it has also been linked to mental health. Accumulating evidence from animal and adult human studies has uncovered several key bidirectional communication pathways between the gut microbiota and brain functioning, named the microbiota-gut-brain axis (MGBA) (Cryan et al., 2019). Remarkably, the MGBA is not only functional in adults, but starts playing an equally or even more important role at early ages with regard to child behavior and cognition (Cryan et al., 2019). Both the gut microbiota and the brain develop at a breathtaking pace during early life, however, only few studies investigated associations between the gut microbiota and behavior in such sensitive periods. Therefore, this study aimed to investigate the relations of the gut microbiota in the first three years of life with child problem behavior and executive functions at the age of three years.

The bidirectional interactions of the MGBA occur through intricately innervated and highly adaptable neuronal pathways, and extremely delicate and difficult-to-measure molecular communication systems (Cryan et al., 2019; de Weerth, 2017). For instance, short chain fatty acids (SCFAs), mainly being produced through dietary fiber fermentation by the gut microbiota, likely affect the brain via the vagus nerve, immunity, and the endocrine system (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019). Furthermore, specific microbial taxa can generate γ-aminobutyric acid (GABA), which is the main inhibitory neurotransmitter of the central nervous system and regulates many physiological functions (Mazzoli & Pessione, 2016; Silva, Bernardi, & Frozza, 2020). The symporter that mediates the uptake of microbiota-derived GABA is present through the gastrointestinal tract, suggesting that luminal GABA is able to cross the gut barrier and influence extra-gut targets. Although remaining controversial, recent studies suggest the permeability of the blood-brain barrier to GABA, implying its direct impact on the central nervous system (Mazzoli & Pessione, 2016). Besides, GABA receptors are widely expressed in enteric neurons and immune cells, indicating the role of GABA in regulating the gut-to-brain signaling and neuroinflammation (Auteri, Zizzo, & Serio, 2015; Hyland & Cryan, 2010). Such pathways along the MGBA may partially explain how the gut microbiota impacts mental health.

The colonization of the gut by microorganisms mostly commences soon after birth and continues in the following years. Gut microbial disturbances during this dynamic and sensitive period can result in subsequent health problems, such as developing allergies and obesity (Zhuang et al., 2019). This is explained by the early life programming theory, that refers to long lasting changes and disruptions as a consequence of environmental exposures at a young age (Tarry-Adkins & Ozanne, 2011). In early life, the brain experiences numerous quick developments in neuronal proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis (Rice & Barone, 2000), largely impacting brain functioning, cognition and behavior (Erus et al., 2015). Simultaneously, the microbiota is becoming established in the gut of infants and young children (de Weerth, 2017; S. Wang et al., 2018).

Thus, alterations of the gut microbiota in early life may exert considerable effects on the development of the brain. Indeed, there is compelling evidence from animal studies supporting such a hypothesis (Clarke, O'Mahony, Dinan, & Cryan, 2014; Leclercq et al., 2017; O'Mahony et al., 2014; Stilling et al., 2015). This marks early life a sensitive time window to obtain and maintain microbiota composition that will promote normal physical and mental development. However, we know little about early-life gut microbiota in association with child behavior and cognition. Specifically, how the gut microbiota and brain functioning, in particular host behavior, are interconnected in low-risk community infants and children (i.e., generally healthy and neurotypically developing) is underexplored. Knowledge on these associations, particularly when uncovered by comprehensive longitudinal studies, can provide insight into the typical early development of the gut microbiota in relation to child behavior and cognition.

First studies have found evidence for associations between the gut microbiota and child behavior and cognition. Regarding behavior, Loughman et al. reported that increased relative abundances of taxa belonging to the genus *Prevotella* at one year of age were associated with less internalizing behavior at age two (i.e., problem behavior affecting internal psychological conditions, characterized by withdrawal, anxiety, and emotional problems (T. M. Achenbach, 1966)) (Loughman et al., 2020). In our previous study, we found that the rise of *Prevotella* 9 in middle childhood was related to more externalizing behavior at age ten (i.e., problem behavior exhibited in the external environment, including features like impulsivity, aggression and hyperactivity (T. M. Achenbach, 1966)) (Ou, Belzer, Smidt, & de Weerth, 2022). Besides, Laue et al. observed a negative relation between *Streptococcus peroris* and internalizing behavior in girls before school age, and a positive association between *Lachnospiraceae* species and externalizing behavior in both genders (Laue et al., 2021). Furthermore, *Lachnospiraceae* species and *Veillonella* were linked to more internalizing behavior in pre-schoolers; interestingly, *Veillonella* was positively related to externalizing behavior as well (Van De Wouw et al., 2022). Additionally, increased alpha diversity was observed in preschool children with less internalizing (Laue et al., 2021; Van De Wouw et al., 2022).

Four other studies have found an underlying link between infant gut microbiota and child cognition (Aatsinki et al., 2020; Carlson et al., 2017; Rothenberg et al., 2021; Streit et al., 2021; Tamana et al., 2021). Cognition is fundamental for the development of executive functions, including higher-level cognitive processes like inhibitory control (Diamond, 2013). Specifically, a cross-sectional study found more *Enterobacteriaceae* species in relation to worse cognition at age 45 months (Streit et al., 2021). Longitudinal research reflected that high relative abundances of *Bacteroides* at age one year were related to better cognition at age two (Carlson et al., 2017; Tamana et al., 2021). Furthermore, *Faecalibacterium* at one year of age was associated with reduced cognitive functions at age two (Carlson et al., 2017). Additionally, a lower relative abundance of *Bifidobacterium* and a higher relative abundance of *Clostridium* at two-and-a-half months were linked to increased attention at eight months (Aatsinki et al., 2020). Moreover, Rothenberg et al. found that children with better cognition showed enriched *Faecalibacterium*, *Sutterella* and *Clostridium* cluster XIVa at age three years.

Finally, high alpha diversity at age one year was reported in two-year-old children with worse cognition (Carlson et al., 2017).

To conclude, a number of associations have been observed between the gut microbiota and problem behavior and cognition in early life, but findings are variable and inconsistent across studies. Furthermore, most of the previous studies have assessed problem behavior and cognition by using only one questionnaire of a single reporter. In the current longitudinal study in a community sample of children, we investigated the gut microbiota in relation to problem behavior (i.e., internalizing and externalizing behavior) and executive functions (i.e., advanced cognitive abilities, including inhibitory control (Diamond, 2013)) using questionnaires of multiple reporters and behavioral tasks. We had the following two hypotheses: (1) relative abundances and alpha diversity (i.e., Chao1, Shannon, and phylogenetic diversity) of the gut microbiota at age three years are associated with reported problem behavior and executive functions at the same age; (2) relative abundances and alpha diversity of the gut microbiota at early ages (i.e., two, six, and 12 weeks, and one year) are associated with reported problem behavior and executive functions at age three.

We investigated these hypotheses in three ways: (1) as the gut microbiota is highly dynamic in early life, its composition at different ages may be differently associated with problem behavior and executive functions later in life. For this reason, we analyzed the overall gut microbiota composition in relation to preschool-aged cognitive measures in an age-specific manner; (2) for the same reason, relations regarding a single taxon and an alpha diversity index were analyzed in an age-specific manner; (3) based on the age-specific analyses, we explored the trajectories of taxa and alpha diversity parameters in association with mental outcomes over the whole study period. Figure 1 shows the workflow of our analyses. Considering that most published findings were at the genus level, we performed our analyses at the same taxonomic level. However, given the paucity of studies on these relations at such early ages, we did not hypothesise specific associations between microbial taxa and mental outcomes.

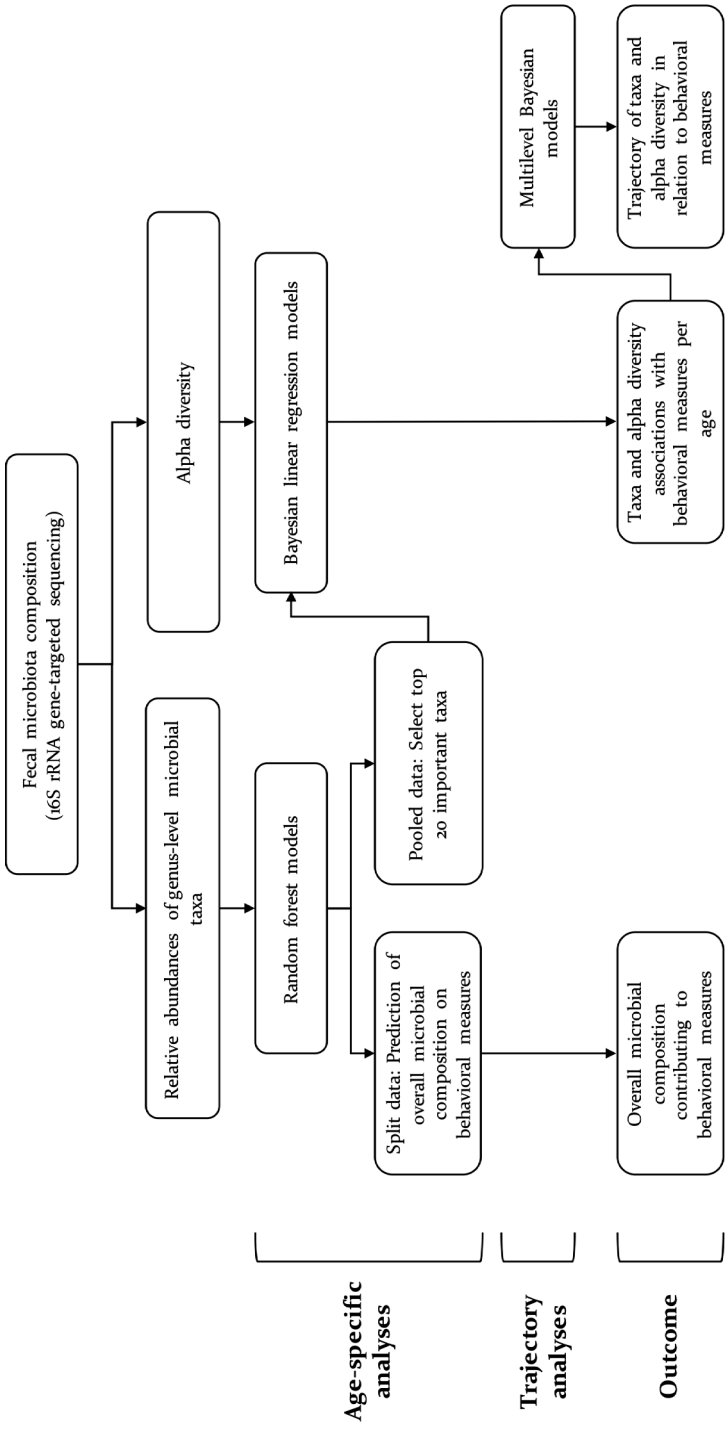

Figure 1. Workflow of the analyses.

Materials and Methods

Participants

The current study is part of the longitudinal Dutch study named *BINGO* where early factors associated with child development were investigated. Participants were healthy children and their parents living in the Netherlands. Detailed in- and exclusion criteria are described in a previous publication (Hechler, Beijers, Riksen-Walraven, & de Weerth, 2018). At baseline, 88 pregnant women joined the *BINGO* study. Postnatal exclusion criteria included: complications during pregnancy, gestational age at birth <37 weeks, birth weight <2500 g, 5-min Apgar score <7, and congenital malformations. Seventy-seven mothers were followed up after postnatal exclusion. At child age three years, 76 families were approached (one drop-out occurred during the previous measurement rounds). Among them, two families could not be contacted, six families did not participate due to time constraints, and one family dropped out due to personal reasons. Parental demographics did not differ significantly between participating and non-participating families. This resulted in a final sample of 67 families. Of them, 64 families participated in home visits when their children reached age three, and the other three families were unable to join home visits but filled out questionnaires in this assessment round. Both parents participated in 54 families (81%, 54/67), and only mothers participated in 13 families (19%, 13/67).

Ethics

The *BINGO* study was independently reviewed by the Ethics Committee of Social Sciences of Radboud University, and no formal objection to this research was made [ECSW2014–1003–189 and amendment: ECSW–2018–034]. The current study was preregistered on the Open Science Framework: https://osf.io/vwgef with amendment: https://osf.io/nyeb4.

Data Collection Procedure

Collection of child stool samples was done at the ages of two, six and 12 weeks, and one and three years. Stool samples were stored in the participant's freezer (-20°C) until they were collected with a portable freezer. The stool samples were stored at -80°C at Radboud University prior to being processed at the Laboratory of Microbiology at Wageningen University & Research.

Home visits took place at child age three years. Prior to the home visit, mothers and their partners independently filled in digital questionnaires about their child's problem behavior and executive functions. During the home visit, the child performed inhibitory control tasks. Tasks were video recorded and afterwards rated by two trained observers.

Measures

Gut microbiota composition

Stool samples were collected with a polystyrene 10 mL stool container. Total DNA was extracted from 0.01-0.15 g of stool sample with 300 µL of Stool Transport and Recovery Buffer by double bead-beating steps as previously described (Gu et al., 2018). The variable V4 region of prokaryotic 16S ribosomal RNA (rRNA) genes was then amplified by PCR in duplicate reactions, by using primers 515F-n (5'-GTGCCAGCMGCCGCGGTAA) and 806R-n (5'-

GGACTACHVGGGTWTCTAAT) (Gu et al., 2018). The 16S rRNA gene sequencing was completed on the Illumina HiSeq platform by Eurofins Genomics Germany GmbH.

Behavioral measures

Parental questionnaires

Mothers and their partners filled in all questionnaires mentioned below. However, because fewer partners completed the questionnaires, we used partner reports for sensitivity analyses to validate the maternal reports by calculating Kendall correlations between both. The non-parametric Kendall method was chosen due to its better performance in handling non-normally distributed data and tied values (Kendall, 1945). Maternal reports were used as the final measure of reported problem behavior and executive functions.

To assess child problem behavior, the Child Behavior Checklist for ages of one and a half to five years (CBCL, 103 items) (Thomas M. Achenbach & Ruffle, 2000) and the Strengths and Difficulties questionnaire (SDQ, 25 items) (Goodman, 1997) were used. The CBCL and the SDQ include internalizing and externalizing subscales, consisting of items scored on a three-point Likert scale. The SDQ can detect problem behavior as accurately as the CBCL does (Goodman & Scott, 1999). Given that the Kendall correlations on the same subscales of the CBCL and the SDQ were lower than 0.5 (Table S1), we included both instruments separately in the analyses. In both instruments, higher scores on subscales indicate more problem behavior.

To evaluate child executive functions, the Behavior Rating Inventory of Executive Function – preschool version (BRIEF-P, 63 items) questionnaire for pre-schoolers (Sherman & Brooks, 2010) and the Ratings of Everyday Executive functions (REEF, 77 items) (Nilsen, Huyder, McAuley, & Liebermann, 2017) were used. The BRIEF-P and the REEF are scored on three- and four-point scales, respectively. A higher score on the BRIEF-P indicates worse executive functions, while a higher score on the REEF indicates better executive functions. The BRIEF-P is a commonly used questionnaire that measures general executive functions and does not differentiate between different situations. The REEF rates executive functions in different situations (e.g., executive functions around friends, during grocery shopping, or in the community) and determines an average score. Kendall correlations between the BRIEF-P and the REEF were lower than 0.5 (Table S1), hence both instruments were included in the analyses.

Parental questionnaires were considered acceptable and reliable based on their ω_{total} (ranged between 0.65-0.94) or Cronbach's α values (ranged between 0.83-0.96) (Table S2) (Revelle & Condon, 2019).

Inhibitory control tasks

Six different behavioral tasks with good reliability (i.e., Flanker, Whisper, Gift Wrap, Gift Delay, Snack Delay, and Bear Dragon) were performed to measure inhibitory control as previously stated in detail (Willemsen, Beijers, Arias Vasquez, & de Weerth, 2021). Snack Delay and Bear Dragon were excluded from the analyses due to insufficient variation and low number of children that passed the practice trials, respectively. The other four tasks were included in our study. Higher scores on these tasks indicate better inhibitory control.

Statistical analyses

Pre-processing of sequence data

Sequence data were processed via *NG-Tax* 2.0 with default settings (Poncheewin et al., 2020; Ramiro-Garcia et al., 2018), with SILVA SSU 16S rRNA gene reference database (version 132) (Quast et al., 2012). The raw amplicon sequence variant (ASV) count data were used to calculate alpha diversity by the *ape* (Paradis, 2020) and the *picante* (W. Kembel, 2020) packages. Then, ASV count data were glommed at the genus level prior to analyses.

Gut microbiota composition and development over the first three years of life

For descriptive purposes, we first delineated gut microbiota composition and development in the first three years after birth (including all samples at the age of two, six and 12 weeks, and one and three years). We compared differences in alpha diversity indices, including Chao1, Shannon, and phylogenetic diversity, between ages using Wilcoxon rank-sum tests corrected with the False Discovery Rate (FDR) method. Next, we also compared beta diversity between ages by conducting Principal Coordinate Analysis (PCoA) via the *vegan* package (Oksanen, n.d.). Considering that PCoA can be applied to different dissimilarity and distance metrics that all differ in specific aspects and corresponding interpretation, we included the Bray-Curtis, weighted Jaccard (formula = 2*Bray-Curtis dissimilarity / (1 + Bray-Curtis dissimilarity)), unweighted UniFrac, weighted UniFrac, and Aitchison (the Euclidean distance based on centered-log-transformed ASV count data) methods, to comprehensively describe the compositional differences. Except for the Aitchison distance, we transformed genus-level count data into relative abundances before calculating other dissimilarity and distance metrices. Significance was determined as a p value lower than 0.05 for non-multiple tests and an FDR-adjusted p value lower than 0.05 for multiple tests.

Additionally, we visualized average and individual relative abundances at the genus level over the study period by using a barplot and a heatmap, respectively. To identify differentially abundant microbial taxa at the genus level between ages, we conducted the Linear Discriminant Analysis Effect Size (LEfSe) method by using the *microbiomeMarker* R package (Segata et al., 2011), with a log-transformed Linear Discriminant Analysis (LDA) score higher than two indicating significance.

Confounding effects

In our original preregistration, we considered child age and diet quality as potential confounders (i.e., variables that influence both the independent variables and the outcome). After reconsideration, both variables were removed as potential confounders due to two major reasons (amendment can be found via https://osf.io/nyeb4): (1) low variation in child age (see Figure 2 for notes regarding ages); (2) our previous study using the same cohort found no significant associations of diet quality with behavior and executive functions (Willemsen et al., 2021). Potential covariates of the independent variables only (i.e., the gut microbiota) were not accounted for in downstream analyses (Cinelli, Forney, & Pearl, 2020), as they would remove variation in the gut microbiota data, which was not the purpose of this study. These potential confounders and covariates as well as their relations to the gut

microbiota and behavioral outcomes are displayed in a directed acyclic graph (DAG) (Figure S1).

Data imputation and transformation

Missing values (proportion of missing values is shown in Table S3) in problem behavior, executive functions, and inhibitory control were imputed ten times together, by using the predictive mean match (PMM) method in the R package *mice* (Buuren, 2021). The imputation model was conducted separately at each age. For instance, at the age of three, 64 children provided gut microbiota data, and their missing values in aforementioned behavioral measures were imputed jointly in one model. No auxiliary variables (i.e., variables that are not included in analyses, but are correlated with imputed variables) were considered in the imputation.

For both random forest models and the Bayesian linear regression models, genus-level relative abundance data were used. Numeric variables were standardized (i.e., subtracting the mean and dividing by the standard deviation) for the Bayesian models only, as random forest models rely on decision trees for which standardization is considered unnecessary.

Main analyses

To determine whether gut microbiota composition in the first three years of life (i.e., two, six and 12 weeks, and one and three years) is associated with problem behavior (i.e., internalizing and externalizing behavior) and executive functions (including inhibitory control) at age three, we conducted random forest models and the Bayesian linear regression models (Bürkner, 2017; Kuhn et al., 2020). Random forest is first of all well suited to analyse microbiome data as it is appropriate for high dimensional data, invariant to scaling of inputs, computationally efficient, and able to uncover nonlinear relationships (Belk et al., 2018; Louppe, 2014; Namkung, 2020). The first random forest model was applied to assess the contribution of the total gut microbiota composition on our behavioral outcomes. This was done for the purpose of exploring the gut microbial community as a whole to account for the complex interplay between taxa. The following random forest model was applied as a preselection tool, to select possibly important taxa from high-dimensional data, before passing them on to the Bayesian linear regression model. The Bayesian model was first used to determine age-specific relations (i.e., directions and strengths) of a selected taxon and an alpha diversity index with each outcome measure. By looking at the different time points separately, these analyses can help identify periods of development that are sensitive to certain microbial compositions. Although not preregistered, after reconsideration, we decided to perform an additional analysis to optimize the use of our longitudinal data. Based on the age-specific results, we implemented a multilevel Bayesian model to determine whether trajectories of change in the gut microbiota were associated with the outcome measures at age three. Figure 1 shows the workflow of our analyses.

Age-specific analyses

Determining the contribution of the overall gut microbiota to each behavioral measure through random forest models

Data were imputed ten times: data were randomly split into a training dataset (including 80% participants) and a testing dataset (including 20% participants), leading to ten training datasets and ten corresponding testing datasets. The procedure of data splitting was applied to children who provided gut microbiota information at each age separately. To prevent data leakage, the missing values of behavioral measures were imputed in training and testing datasets separately (ten times) as described earlier. Next, we included genus-level relative abundances of overall gut microbiota as independent variables and one behavioral measure as an outcome. This step was performed on each individual behavioral measure separately. To train the model, a ten-repeated ten-fold cross-validation was conducted on each complete training dataset including imputed values via the *caret* package (Kuhn et al., 2020). Afterwards, we used the trained model to obtain predicted behavioral outcomes of each corresponding complete testing dataset including imputed values. Similarity between predicted and actual behavioral outcomes of the complete testing dataset was measured by the Pearson correlation with its *p* value obtained from a permutation test (N = 1000). Considering that data splitting and imputation resulted in multiple datasets, we used the median value of the Pearson correlation coefficient from multiple cases to represent the final similarity. The *p* value corresponding to this median was included. *P* values were adjusted with FDR methods, with corrected values under 0.05 indicating significance.

Preselecting potentially important gut microbiota contributing to each behavioral measure through random forest models

To identify microbial taxa that contribute to each behavioral outcome, we measured the change in the generalized cross-validation (GCV) value in the random forest model. Larger GCV changes indicate more contribution of the independent variable to the model, in other words, this analysis shows which taxa are potentially more important (Kuhn et al., 2020). Unlike the first random forest model, we did not split the data but used the whole dataset here, because we prioritized the structure of the model and a large sample size can provide more valid information. Missing values of behavioral measures in the whole dataset were imputed as described in the section of data imputation and transformation. Then, relative abundances of all taxa were treated as independent variables with one behavioral measure as an outcome. This procedure was performed on each behavioral measure separately. Next, we carried out a ten-repeated ten-fold cross-validation on each complete dataset containing imputed data and calculated average GCV values of multiple datasets acquired from data imputation. Based on the size of average GCV values, we selected the largest 20 taxa as the top 20 in importance. These 20 taxa were then passed to the Bayesian linear regression models to confirm their actual associations with behavioral measures.

Associating the gut microbiota with behavioral measures by using Bayesian linear regression models

We implemented Bayesian linear regression models to estimate the relations of relative abundances of the selected top 20 microbial taxa with a prevalence value higher than 10% and alpha diversity with the child behavioral measures. Compared to standard linear regression models, the Bayesian linear regression models compute the probability of different effects rather than simply reporting single estimates of the "true effect" (Bürkner,

2018). We performed the Bayesian models by using the *brms* R package built based on the programming language *Stan* (Bürkner, 2017). The *brm* function within the *brms* package was used with the Gaussian distribution (mean = 0, std = 1) as the prior distribution for all beta coefficients and the Student's t-distribution for error distribution (due to better performance in handling extreme values) (Lange, Little, & Taylor, 1989). A list containing multiple complete datasets including imputed data was directly passed to the *brm* function, which in turn generated a single estimate. Other arguments of the *brm* function were set as follows: chains = 4, iter = 2000, and warmup = 1000. Under these settings, chains were converged properly with Rhat values lower than 1.01. Regarding the outcomes of the Bayesian models, the less the posterior distribution overlaps with zero, the more likely a relation is positive or negative. In the current study, we defined a relation as positive or negative with confidence when its 95% credible interval (CI) excluded the value zero.

Trajectory analyses

Relating the developmental trajectories of the gut microbiota to behavioral measures through multilevel Bayesian linear regression models

To make maximum use of our longitudinal data, we conducted multilevel models to investigate relations between the developmental trajectories of the gut microbiota and behavioral measures. The multilevel models were performed on microbial taxa and alpha diversity with confident age-specific relations to behavioral measures (i.e., as determined by the Bayesian linear regressions described above). In the multilevel models, microbial and behavioral information as well as the actual age were level 1 variables, and the child was the level 2 variable. Note that missing values in behavioral measures and actual age were not imputed, and that in these analyses we used the same distributions and arguments as described earlier. Before performing a testing model, we first checked the intraclass correlation (ICC) of an intercept-only model. When the 95% CI of an ICC excluded the value zero in the intercept-only model, multilevel strategies were used. A trajectory relation was considered with confidence when there was no
overlap between its 95% CI and zero.

With respect to taxa, when their prevalence was higher than 10% at five time points (i.e., two, six, and 12 weeks, and one and three years), multilevel models were performed on the pooled data of all ages. When only the first three time points met the 10% criteria, multilevel models were carried out by pooling samples at these three ages together. When only the prevalences at the last two ages were higher than 10%, multilevel approaches were done in the pooled aged-one-and-three years samples. Rhat values were used to check chain convergence.

Results

Population characteristics and descriptives

Demographic data and descriptives of study variables are shown in Table 1. Roughly 50% of the children were girls. Mothers were mostly highly educated (86.2%). Scores on the questionnaires measuring child problem behavior and executive functions did not differ

significantly between mothers and their partners, and they were significantly positively intercorrelated (Table S1).

Table 1. Descriptives of study subjects.

	Characteristics						
Categorical variable	Ratio						Sample size
Child sex	girl: boy = 34:30						64
Delivery mode	vaginal: C-section= 54:7						61
Educational level (%)	low: middle: high = 0:12.5:87.5						64
Numeric variable	Mean ± SD	Minimum	Lower quartile	Median	Upper quartile	Maximum	Sample size
Age at age three in years	3.2 ± 0.1	3.1	3.1	3.2	3.2	3.5	63
Gestational age in weeks	39.8 ± 1.5	35.6	38.9	40	40.9	42.1	63
Birth weight in grams	3556 ± 426.2	2570	3270	3480	3885	4445	61
Total breastfeeding duration in months	9.6 ± 8.1	0	4	8	13.2	36	64
Total exclusive breastfeeding duration in months	3.9 ± 1.7	0	3	4	5	7	53
Age at solid food introduction in months	4.6 ± 1	3	4	4	5	7	59
Average diet quality at age three	4 ± 1.2	2	3.3	3.9	4.8	7.2	64
CBCL_M_Internalizing	7.1 ± 5.7	0	3	5.5	10.8	24	62
CBCL_M_Externalizing	11.8 ± 7.3	0	7	12	15.8	31	62
SDQ_M_Internalizing	3.6 ± 2.5	0	2	3	5	11	62
SDQ_M_Externalizing	5.5 ± 3	1	3.2	5	7	14	62
BRIEF-P_M_TotalScore	94.4 ± 15.4	69	83	92	106.5	146	63
REEF_M_TotalScore	151.1 ± 31	74	133.2	153	172.8	215	62
Flanker	1.6 ± 0.3	0.9	1.4	1.7	1.9	2	47
Whisper	1.9 ± 0.3	0.9	1.8	2	2	2	60
Gift Wrap	2.2 ± 0.9	0	1.5	2.5	3	3	60
Gift Delay	3.9 ± 0.2	2.9	3.9	4	4	4	61
CBCL_P_Internalizing	7.5 ± 5.5	0	4	6	11	22	49
CBCL_P_Externalizing	12.2 ± 5.8	1	8	12	17	24	49
SDQ_P_Internalizing	3.5 ± 2.4	0	2	3	5	9	44
SDQ_P_Externalizing	5.4 ± 2.9	0	3	5	7	12	50
BRIEF-P_P_TotalScore	97.2 ± 17.8	69	86.2	96	109.2	137	50

REEF_P_TotalScore	147 ± 28.9	78	133	148	164	212	49

Notes. In the assessment round at age three, 64 participants were included in the study. In total, 66, 70, 73, 72, and 64 fecal samples were collected at ages two, six, and 12 weeks, and one, and three years, respectively. P, Partner; M, Mother; CBCL, the Child Behavioral Checklist; SDQ, the Strengths and Difficulties Questionnaire; BRIEF-P, Behavior Rating Inventory of Executive Functions - Preschool; REEF, Ratings of Everyday Executive Functioning. Differences were compared between mother and partner reports by Wilcoxon tests. None of them were significant before or after FDR adjustments.

Gut microbiota composition and development over the first three years of life

We analyzed microbial composition of 345 fecal samples taken at five time points. A total of 42,056,591 high-quality reads were obtained after being processed with *NG-Tax* 2.0. Within these reads, 220 microbial taxa were identified at the genus level mainly belonging to the phyla *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia*.

For descriptive purposes, we compared alpha and beta diversity between ages (Figures 2; beta-diversity comparisons between the first three ages and between the last two ages are displayed in Figures S2 and S3) and delineated a general developmental trajectory of the gut microbiota over time (Figure 3a). Diversity comparisons reflected profound compositional differences between infancy and toddlerhood. These differences were visualized by the heatmap showing individual relative abundance data (Figure S4). LEfSe identified a total of 106 differentially abundant microbial taxa between ages (log-transformed LDA scores higher than two; Table S4). Due to the large number of significant taxa, only the taxa with log-transformed LDA scores higher than four are highlighted and displayed in Figure 3b, such as an unidentified genus within *Enterobacteriaceae*, *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, and *Blautia*.

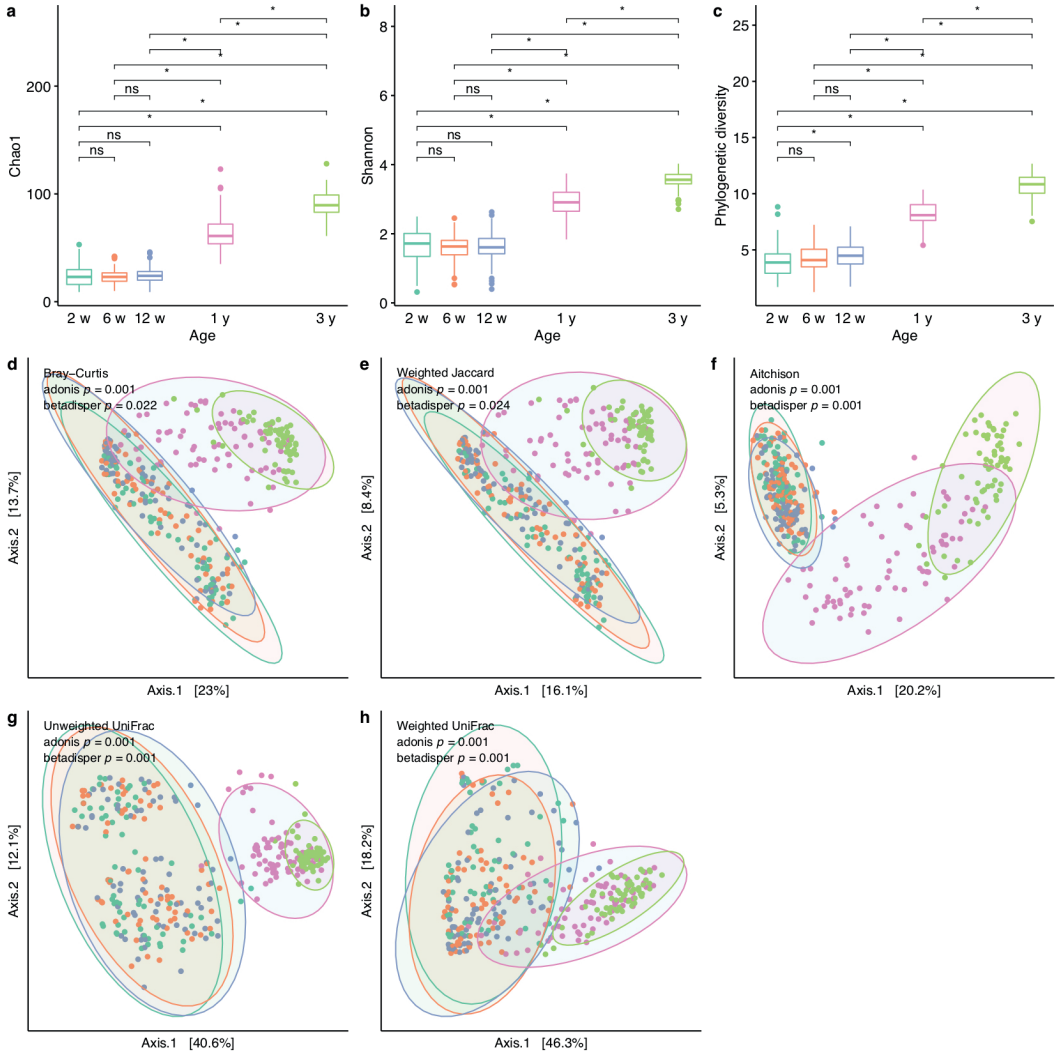

Figure 2. Alpha and beta diversity of the gut microbiota in the first three years of life. (a-c) Alpha diversity as measured by Chao1, Shannon, and phylogenetic diversity indices. Wilcoxon rank-sum tests were conducted between ages and corrected with the FDR method (ns, not significant; *, <0.01). $Age_{2w\_mean\pm sd}$ = 2.08 ± 0.28. $Age_{6w\_mean\pm sd}$ = 6.23 ± 0.55. $Age_{12w\_mean\pm sd}$ = 12.27 ± 0.42. $Age_{1y\_mean\pm sd}$ = 1.04 ± 0.08. $Age_{3y\_mean\pm sd}$ = 3.18± 0.10. (d-h) Principal coordinate plots of beta diversity, based on different pairwise dissimilarity (Bray-Curtis and weighted Jaccard) and distance (UniFrac and Aitchison) matrices, with points and ellipses colored by ages (Lake blue, two weeks; Orange, six weeks; Purple, 12 weeks; Pink, one year; Grass green, three years).

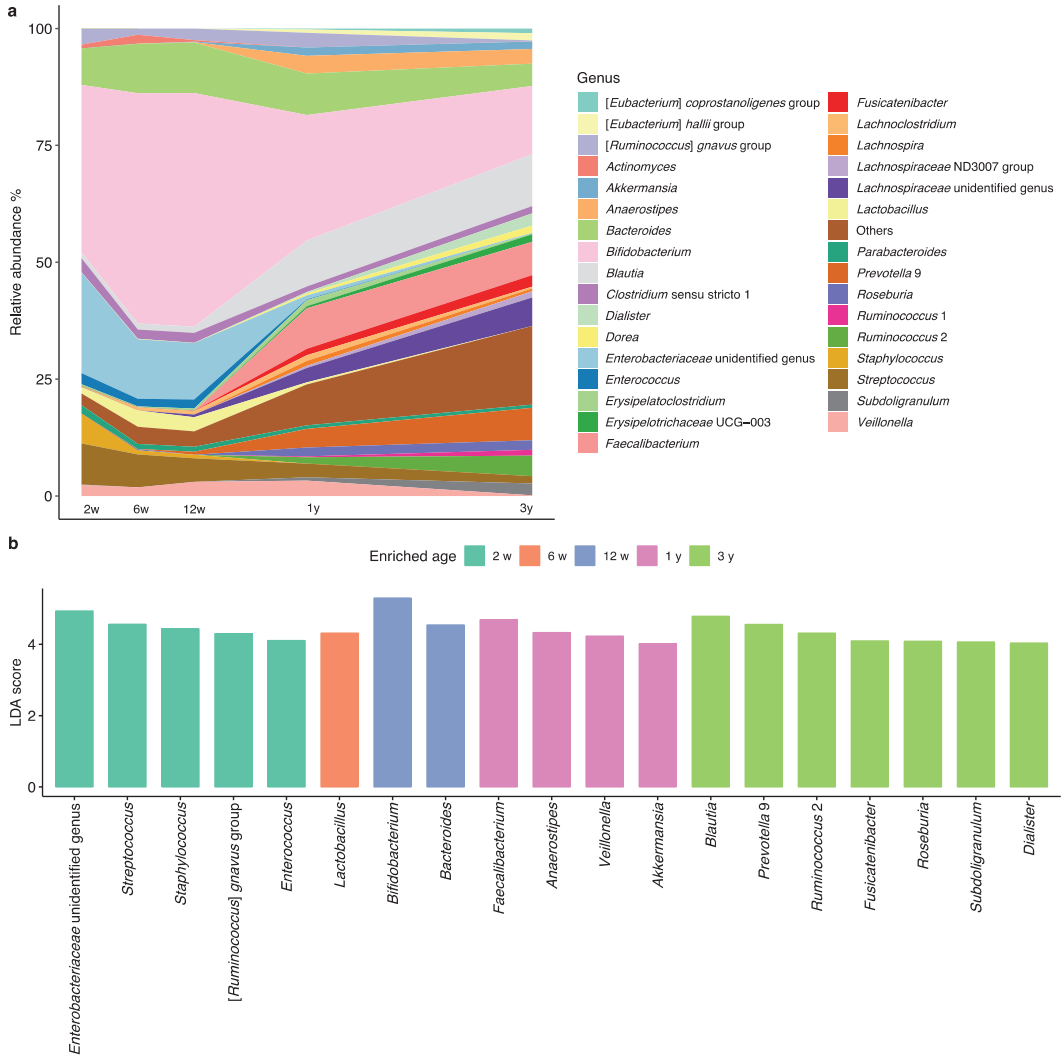

Figure 3. Characteristics of the gut microbiota in the first three years of life. (a) Average relative abundances of the gut microbiota at the genus level over time. Others represent genera with relative abundances lower than 1%. (b) Differentially abundant genus-level taxa between ages, identified by Linear Discriminant Analysis Effect Size (LEfSe) with log-transformed Linear Discriminant Analysis (LDA) scores higher than four.

Age-specific analyses

Determining the contribution of the overall gut microbiota to each behavioral measure through random forest models

To explore whether the overall microbial composition in the first three years (i.e., at ages two, six and 12 weeks, and one and three years) contributes to problem behavior and executive functions at age three, we compared the similarity between the actual and the

predicted behavioral results. As shown in Table S5, 92% (46/50) of the models showed insignificant absolute correlation coefficients (i.e., lower than 0.3), indicating a low likelihood that the gut microbiota can contribute to behavioral outcomes. Regarding the 8% (4/50) models with correlation coefficients higher than 0.3, the similarity remained insignificant between the actual and predicted data, implying the same low likelihood. The random forest models showed that the overall gut microbiota did not contribute to problem behavior and executive functions in the present study.

Preselecting potentially important gut microbiota contributing to each behavioral measure through random forest models

As planned in our preregistration, we preselected the microbial taxa that may contribute to the behavioral outcomes the most (i.e., top important taxa) based on GCV values, by performing separate random forest models at each age. The top 20 important taxa at the genus level are depicted in Figure 4 and Figure 5, with the following observations:

(1) *Bacteroides* and *Clostridium* sensu stricto 1 were the most frequent contributors to CBCL internalizing behavior;

(2) *Bacteroides* and *Bifidobacterium* were the most frequent contributors to CBCL externalizing behavior, SDQ internalizing and externalizing behavior, and BRIEF-P executive functions;

(3) *Bacteroides* and *Blautia* were the most frequent contributors to REEF executive functions;

(4) Additionally, *Bacteroides* and *Bifidobacterium* were the most frequent contributors to the behavioral measures of inhibitory control (i.e. Flanker, Whisper, Gift Wrap, and Gift Delay) (Figure 6).

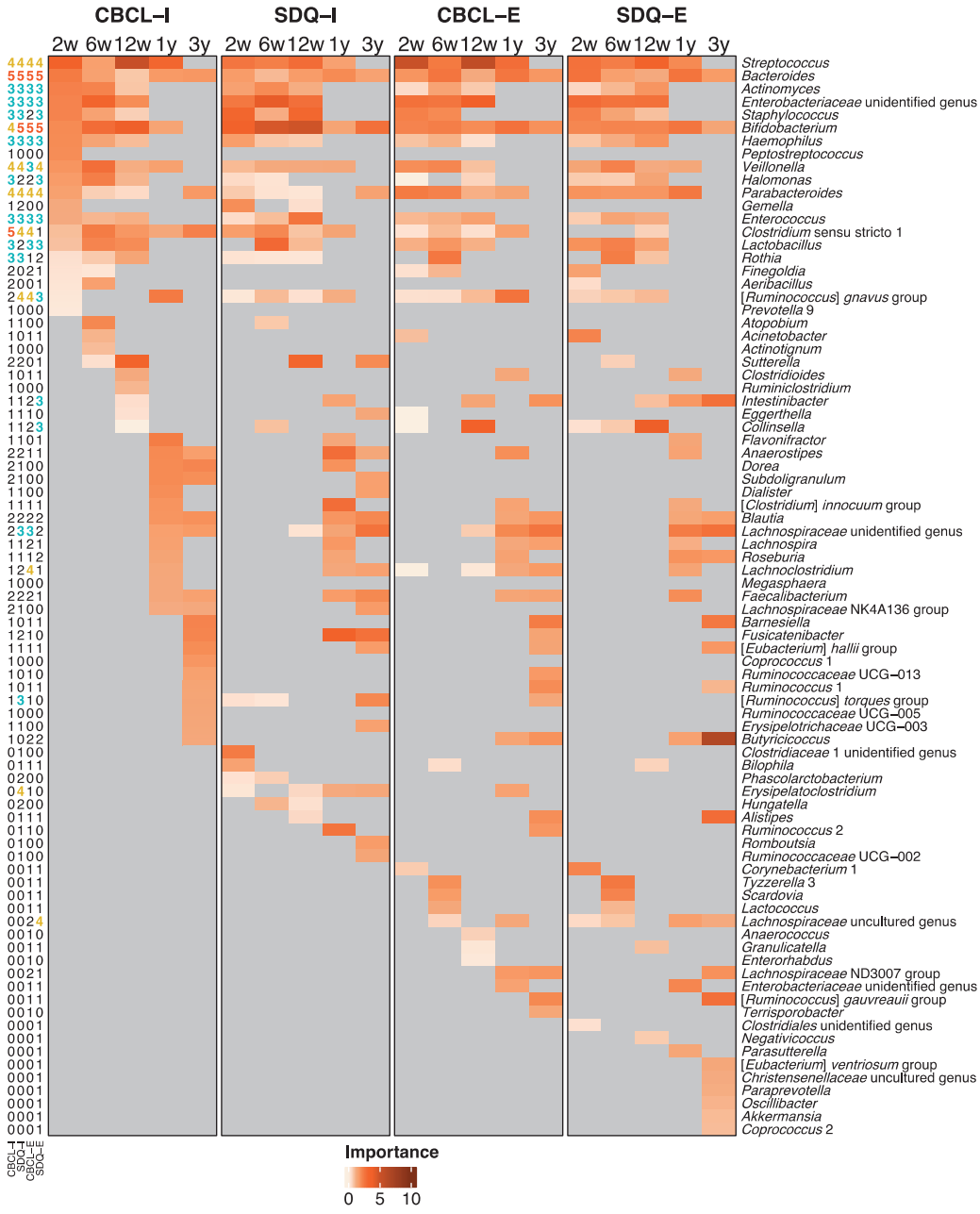

Figure 4. Heatmap showing the top 20 important microbial taxa over time and their associations to problem behavior at age three as reported by the mother. The top 20 important genus-level taxa within each age (i.e., 2w, two weeks; 6w, six weeks; 12w, 12 weeks; 1y, one year; 3y, three years) per behavioral measure are shown on the right side of the figure. Behavioral measures include: CBCL-I, internalizing behavior measured by the CBCL; SDQ-I, internalizing behavior measured by the SDQ; CBCL-E, externalizing behavior measured by the CBCL; SDQ-E, externalizing behavior measured by the SDQ.

The orange scale indicates the importance of the taxa, with darker color referring to increased importance. The importance was determined by the generalized cross-validation value, with a larger value change indicating more contribution of a taxon to the model, i.e., which taxon is more important. As not all taxa appeared in the top 20 list at each time point, these absent taxa are colored in gray. Numbers on the left side of the figure show how many times a taxon appeared to be in the top 20 list of a behavioral measure over time. The frequently appearing taxa are bolded and colored in orange (five times), yellow (four times), or green (three times).

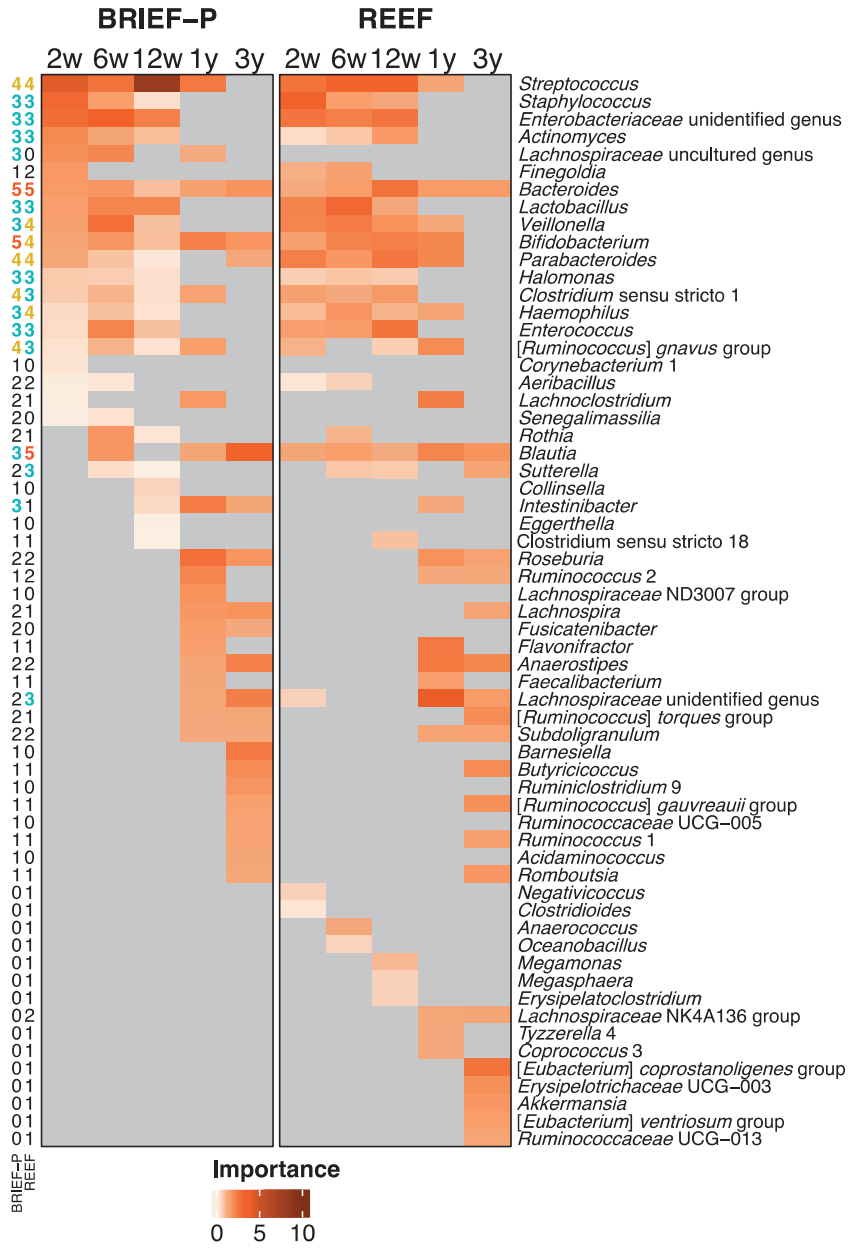

Figure 5. Heatmap showing the top 20 important microbial taxa over time and their associations to executive functions at age three as reported by the mother. The top 20 important genus-level taxa within each age (i.e., 2w, two weeks; 6w, six weeks; 12w, 12 weeks; 1y, one year; 3y, three years) per cognitive measure are shown on the right side of the figure. The measures include: BRIEF-P, executive functions measured by the BRIEF-P; REEF, executive functions measured by the REEF. The orange

scale indicates the importance of the taxa, with darker color referring to increased importance. The importance was determined by the generalized cross-validation value, with a larger value change indicating more contribution of a taxon to the model, i.e., which taxon is more important. As not all taxa appeared in the top 20 list at each time point, these absent taxa are colored in gray. Numbers on the left side of the figure show how many times a taxon appeared to be in the top 20 list of a measure over time. The frequently appearing taxa are bolded and colored in orange (five times), yellow (four times), or green (three times).

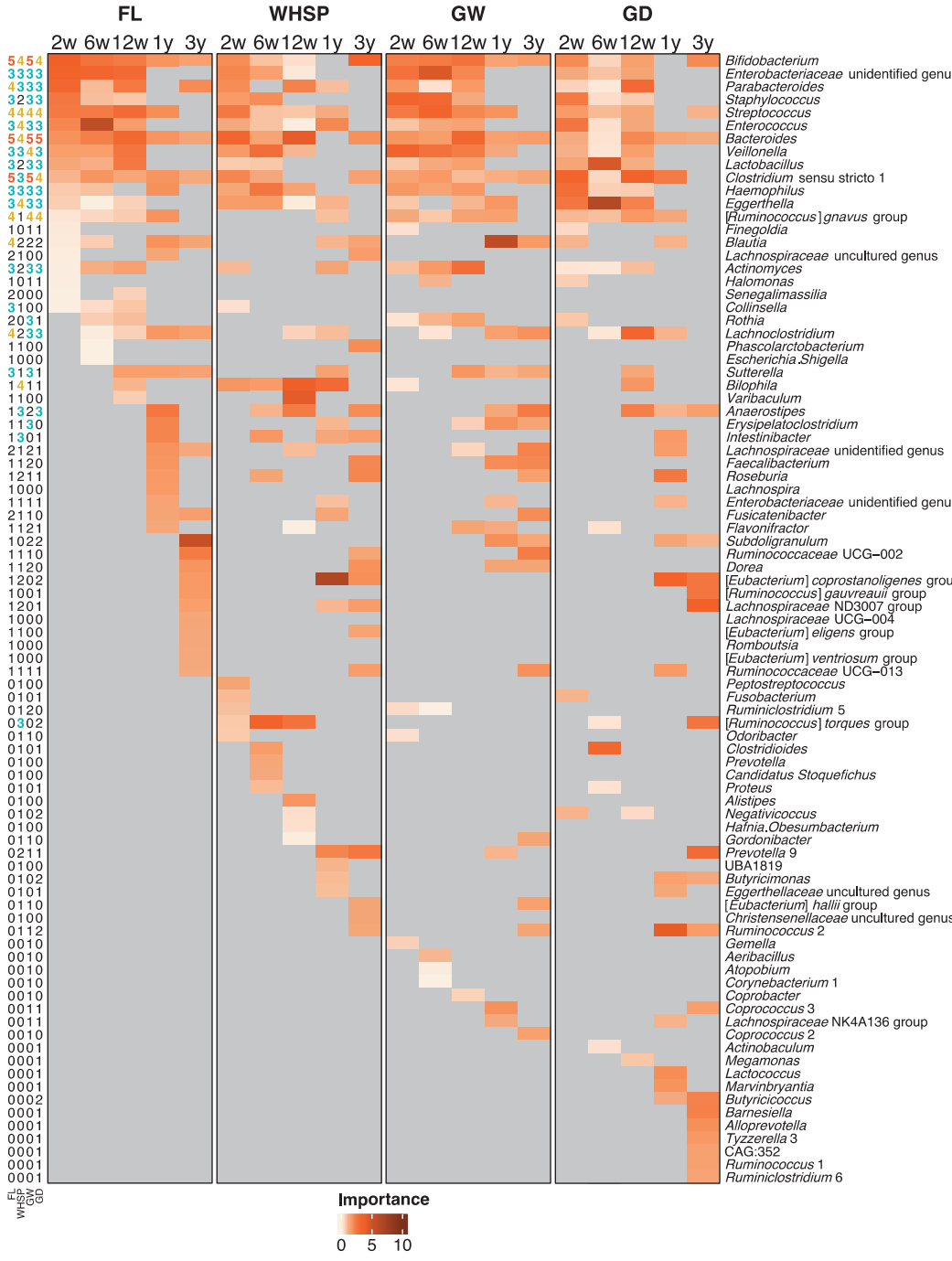

Figure 6. Heatmap showing the top 20 important microbial taxa over time and their associations to observed inhibitory control behavior at age three. The top 20 important genus-level taxa within each age (i.e., 2w, two weeks; 6w, six weeks; 12w, 12 weeks; 1y, one year; 3y, three years) per inhibitory-control task are shown on the right side of the figure. The tasks include: FL, flanker; WHSP, whisper; GW, gift

wrap; GD, gift delay. The orange scale indicates the importance of the taxa, with darker color referring to increased importance. The importance was determined by the generalized cross-validation value, with a larger value change indicating more contribution of a taxon to the model, i.e., which taxon is more important. As not all taxa appeared in the top 20 list at each time point, these absent taxa are colored in gray. Numbers on the left side of the figure show how many times a taxon appeared to be in the top 20 list of a task over time. The frequently appearing taxa are bolded and colored in orange (five times), yellow (four times), or green (three times).

Associating the gut microbiota with behavioral measures by using Bayesian linear regression models

To confirm whether the aforementioned top 20 important microbial taxa were associated with problem behavior and executive functions, we performed the Bayesian linear regression model on each genus-level taxa (relative abundance) and behavior pair. Table 2 shows the strongest observed associations of these pairs (i.e. estimates higher than 0.2 or lower than -0.2).

Table 2. Associations of the gut microbiota in the first three years of life with behavioral measures at age three.

Behavior at age three	Age of the gut microbiota	Genus	Esti mate	Estimate error	95% CI	Preval ence%	Relative abundance %
							mean ± SD
Mother-reported							
CBCL_Inter nalizing	12w	*Intestinibacter*	0.23	0.12	[0.01, 0.47]	16	0.2 ± 0.6
	3y	*Barnesiella*	0.31	0.12	[0.08, 0.55]	50	0.4 ± 0.6
CBCL_Exter nalizing	2w	*Streptococcus*	0.26	0.12	[0.01, 0.5]	94	8.8 ± 10.1
	2w	*Parabacteroides*	-0.3	0.12	[-0.51, -0.06]	35	1.8 ± 4.9
	1y	*Clostridium* sensu stricto 1	0.23	0.11	[0.01, 0.46]	62	1.2 ± 3.5
	1y	*Butyricicoccus*	0.23	0.12	[0.01, 0.48]	56	0.4 ± 0.5
	1y	*Parabacteroides*	-0.22	0.12	[-0.45, -0.01]	44	0.8 ± 1.8
	3y	*Barnesiella*	0.33	0.12	[0.1, 0.56]	50	0.4 ± 0.6
SDQ_Inter nalizing	1y	*Ruminococcus* 2	-0.36	0.11	[-0.58, -0.14]	39	1.4 ± 2.4
	3y	*Bifidobacterium*	0.27	0.13	[0.01, 0.53]	100	14.6 ± 11.6
	3y	*Blautia*	-0.25	0.12	[-0.48, 0]	100	11.1 ± 4.5
	3y	[*Ruminococcus*] *torques* group	-0.25	0.13	[-0.51, -0.01]	84	0.8 ± 0.7
	3y	*Sutterella*	0.25	0.13	[0.01, 0.5]	61	0.3 ± 0.3
SDQ_Exter nalizing	2w	*Enterobacteriaceae* unidentified genus	0.25	0.12	[0.01, 0.5]	89	21.6 ± 24.1
	2w	*Parabacteroides*	-0.28	0.12	[-0.51, -0.05]	35	1.8 ± 4.9
	6w	*Halomonas*	0.28	0.11	[0.06, 0.5]	11	0.1 ± 0.2

	3y	*Butyricicoccus*	-0.35	0.12	[-0.57, -0.11]	89	0.4 ± 0.3
	3y	*Bifidobacterium*	0.27	0.13	[0.01, 0.52]	100	14.6 ± 11.6
	3y	*Oscillibacter*	0.28	0.12	[0.04, 0.51]	22	0 ± 0.1
BRIEF-P	2w	*Streptococcus*	0.4	0.12	[0.15, 0.64]	94	8.8 ± 10.1
	6w	*Halomonas*	0.24	0.12	[0.01, 0.49]	11	0.1 ± 0.2
	12w	*Streptococcus*	0.31	0.12	[0.07, 0.55]	88	5.1 ± 10.4
	12w	*Intestinibacter*	0.3	0.11	[0.08, 0.53]	16	0.2 ± 0.6
	1y	*Ruminococcus* 2	-0.3	0.12	[-0.54, -0.08]	39	1.4 ± 2.4
	1y	*Clostridium* sensu stricto 1	0.27	0.12	[0.03, 0.5]	62	1.2 ± 3.5
	3y	*Blautia*	-0.3	0.13	[-0.57, -0.05]	100	11.1 ± 4.5
REEF	2w	*Parabacteroides*	0.25	0.12	[0, 0.47]	35	1.8 ± 4.9
	6w	*Halomonas*	-0.24	0.12	[-0.48, -0.01]	11	0.1 ± 0.2
	1y	*Lachnospiraceae* unidentified genus	0.28	0.11	[0.06, 0.5]	78	3.1 ± 4.1
	3y	[*Ruminococcus*] *torques* group	-0.24	0.13	[-0.49, -0.01]	84	0.8 ± 0.7
Child-reported							
Flanker	6w	*Bacteroides*	0.28	0.12	[0.05, 0.51]	59	10.6 ± 16.3
	1y	*Anaerostipes*	-0.29	0.12	[-0.51, -0.07]	96	3.8 ± 3.6
	1y	*Sutterella*	-0.24	0.12	[-0.48, -0.01]	46	0.3 ± 0.6
	3y	*Subdoligranulum*	-0.25	0.13	[-0.5, 0]	95	2.5 ± 1.8
	3y	*Ruminococcaceae* UCG-013	0.26	0.12	[0.03, 0.51]	73	0.2 ± 0.2
Gift Wrap	1y	*Subdoligranulum*	-0.31	0.12	[-0.54, -0.08]	31	0.7 ± 1.4
	1y	*Coprococcus* 3	-0.26	0.12	[-0.48, -0.02]	14	0.1 ± 0.3
	1y	*Veillonella*	0.24	0.12	[0.01, 0.47]	71	3.3 ± 5
	1y	*Lachnospiraceae* NK4A136 group	-0.3	0.12	[-0.55, -0.08]	38	0.4 ± 0.7

Notes. Associations of estimates with 95% credible intervals (CIs) excluding 0 are presented. Behavioral measures include: CBCL, the Child Behavioral Checklist; SDQ, the Strengths and Difficulties Questionnaire; BRIEF-P, Behavior Rating Inventory of Executive Functions - Preschool; REEF, Ratings of Everyday Executive Functioning.

Remarkably, there were several highly present taxa (i.e. prevalent in more than 80% of the samples, relative abundance higher than 10%) in relation to the outcome measures: *Bifidobacterium* at age three years was associated with more internalizing and externalizing behavior (est. = 0.27 for both), *Blautia* at three years was linked to less internalizing behavior (est. = -0.25), and an unidentified taxa within the *Enterobacteriaceae* family was related to more externalizing behavior (est. = 0.25).

Next, we checked for consensus between the questionnaires assessing the same construct. For internalizing behavior, there was no consensus between the associations found for the CBCL and the SDQ. For externalizing behavior, more *Parabacteroides* at two weeks was associated with less externalizing behavior in both the CBCL (est. = -0.30) and the SDQ (est. = -0.28). An opposite finding was found for *Butyricicoccus* at one year in relation to more externalizing behavior by the CBCL (est. = 0.23), while at three years, it was associated with less externalizing behavior by the SDQ (est. = -0.35).
Within the CBCL results, *Barnesiella* at age three years was associated with more internalizing (est. = 0.31) and externalizing behavior (est. = 0.33). Within the SDQ results, *Bifidobacterium* at age three years was associated with more internalizing and externalizing behavior (est. = 0.27 for both).

Regarding executive functions, *Ruminococcus* 2 at one year and [*Ruminococcus*] *torques* group at age three years, were associated with better executive functions as measured by the BRIEF-P (est. = -0.30, note that higher scores on the BRIEF-P indicates worse executive functions) and worse executive functions measured by the REEF (est. = -0.24), respectively. Lastly, *Halomonas* at six weeks was associated with worse executive functions as measured by the BRIEF-P (est. = 0.24) and the REEF (est. = -0.24).

Different associations were found for the Flanker and the Gift Delay tasks. For the Flanker, relations were identified at the age of six weeks, and one and three years, while for the Gift Wrap, associations were observed at age one year only. This may be due to a highly dynamic gut microbiota ecosystem in early life, of which composition at different ages may be variously linked to executive functions.

There were some overlapping associations between the questionnaires of problem behavior and executive functions. *Parabacteroides* at two weeks was associated with better executive functions (REEF, est. = 0.25), and less externalizing behavior (CBCL and SDQ, est. = -0.30 and -0.28, respectively). Another consistent result was *Streptococcus* at two weeks in relation to worse executive functions (BRIEF-P, est. = 0.40) and more externalizing behavior (CBCL, est. = 0.26).

We also measured behavioral relations to alpha diversity, including Chao 1, Shannon and phylogenetic diversity by using the Bayesian linear regression models (strongest results are displayed in Table 3). Interestingly, relations were only observed for alpha diversity at age two weeks. Higher Chao 1 values were associated with less internalizing behavior (CBCL) (est. = -0.28). Furthermore, Chao1 values were in positive relation to better executive function performance (REEF and Gift Wrap, est. = 0.31 and 0.43, respectively). Lastly, higher

phylogenetic diversity at age two weeks was also linked to better inhibitory control during the Gift Wrap task (est. = 0.32).

Table 3. Associations of alpha diversity in the first three years of life with problem behavior, executive functioning and inhibitory controls at age three.

Reporter	behavior at age three	Age of the gut microbiota	Alpha diversity	Estimate	Estimate error	95% CI
Mother	CBCL_Internalizing	2w	cha01	-0.28	0.12	[-0.51, -0.04]
	REEF	2w	cha01	0.31	0.13	[0.07, 0.57]
Child	Gift Wrap	2w	cha01	0.43	0.12	[0.19, 0.64]
		2w	PD	0.32	0.12	[0.08, 0.56]

Notes. Associations of estimates with 95% credible intervals (CIs) excluding 0 are presented. CBCL, the Child behavioral Checklist; REEF, Ratings of Everyday Executive Functioning.

Trajectory analyses

Relating the developmental trajectories of the gut microbiota to behavioral measures through multilevel Bayesian linear regression models

Based on the results of age-specific Bayesian models and the 10% prevalence rule applied to microbial taxa (Table S6), we identified 16 pairs (including 12 pairs of taxa and behavioral measures, and four pairs of alpha diversity and behavioral measures) available at all five ages (i.e., two, six, and 12 weeks, and one and three years), three at the first three ages, and 12 at the last two ages (Table S7). Higher relative abundances of *Streptococcus* over the first three years of life were weakly related to worse executive functions reported by the BRIEF-P (est. = 0.05; higher scores on the BRIEF-P indicating worse performance), conforming to earlier age-specific findings. We also found that the trajectory of [*Ruminococcus*] *torques* group from age one to three was negatively related to internalizing behavior (SDQ, est. = -0.22), implying that higher relative abundances were associated with fewer internalizing difficulties during this period. No enduring associations were observed with confidence regarding alpha diversity.

Discussion

In this longitudinal study, we investigated associations of the gut microbiota during early life with problem behavior and executive functions, including inhibitory control, at child age three. Multiple associations with behavior and cognition were found for relative abundances of microbial taxa and alpha diversity throughout the first three years of life, in concordance with the early life programming theory (Tarry-Adkins & Ozanne, 2011). Table S8 provides an overview of microbial taxa and alpha diversity in relation to child behavior and cognition, along with a discussion of the existing literature. Below we discuss the most prominent findings.

We found evidence that increased relative abundance of *Streptococcus,* specifically at the age of two weeks and over the first three years after birth, was associated with worse executive functions at the age of three years. This result indicates that *Streptococcus* might affect cognitive development throughout early life. Considering that relations between early-life relative abundances of *Streptococcus* and behavior and cognition in typically developing children have not been observed in previous literature, we examined studies on microbiota composition in children diagnosed with neurodevelopmental disorders as they mostly have comorbid behavioral and cognitive issues (Schoemaker, Bunte, Espy, Deković, & Matthys, 2014). According to a systematic review, children with ASD (autism spectrum disorder) frequently show an overgrowth of *Streptococcus* (Bundgaard-Nielsen et al., 2020). Although gut microbiota dysbiosis in ASD was seemingly partially attributed to an altered dietary pattern (Li et al., 2022; Welberg, 2022), diet was not correlated to the mental outcomes of our community samples. Regarding gene functions, according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa, Sato, & Kawashima, 2022), *Streptococcus* species contain GABA synthesis and transportation relevant genes (e.g. *gadC*), and are involved in tryptophan metabolism. Particularly, reduced cerebral tryptophan has been associated with decreased cognitive flexibility and memory retrieval (Richard et al., 2009). In addition to *Streptococcus*, both age-specific and trajectory relations were discerned for the [*Ruminococcus*] *torques* group: higher relative abundances at the age of three years and throughout the period from one to three years of age were associated with fewer child internalizing problems. Conversely, one study showed excessive absolute abundances of fecal [*Ruminococcus*] *torques* group in children with ASD (L. Wang et al., 2013) and this taxon was strikingly increased in patients with inflammatory bowel disease (Png et al., 2010). Before speculating further on potential mechanisms, it would be insightful to discover whether similar results are observed regarding the trajectory changes of *Streptococcus* and [*Ruminococcus*] *torques* group in replication studies.

With respect to microbial taxa that only showed age-specific associations, we observed higher relative abundances of *Bifidobacterium* at age three years in relation to more internalizing and externalizing behavior at the same age. In contrast, a systematic review showed decreased *Bifidobacterium* in ASD children compared to neurotypically developing controls (Xu, Xu, Li, & Li, 2019). Besides, supplementing ASD children with a prebiotic galacto-oligosaccharide increased *Bifidobacterium* populations in the gut and alleviated autistic symptoms (Grimaldi et al., 2017). However, opposite roles of *Bifidobacterium* have been described in major depressive disorder (MDD) (Cheung et al., 2019; Knudsen et al., 2021). Such inconsistency also takes place within ADHD studies. Two studies reported that *Bifidobacterium longus* mitigated ADHD (Finegold et al., 2010; Pärtty, Kalliomäki, Wacklin, Salminen, & Isolauri, 2015), while another study found overgrowing *Bifidobacterium* species in ADHD subjects (Aarts et al., 2017). Apart from its ability to produce GABA, *Bifidobacterium* contributes to synthesising phenylalanine, which is a precursor of dopamine and noradrenaline (Kandel, Schwartz, Jessell, & Siegelbaum, 2000). Although several studies found altered *Bifidobacterium* levels in ADHD patients, little is known regarding the exact mechanisms (Biederman & Spencer, 1999; Gizer, Ficks, & Waldman, n.d.; Staller & Faraone,

2007). In addition, in the current study, a higher relative abundance of *Blautia* at the age of three years was related to fewer internalizing difficulties (as well as to better executive functions at the same age). *Blautia* is suggested to play an important role in nutrient absorption and digestion (Eren et al., 2015), and in child gut microbiota development towards a normal adult-like configuration (Hsiao et al., 2014). In line with our findings, depleted *Blautia* was seen in ASD populations aged from two to 18 years old by several studies as concluded in a systematic review (Liu et al., 2019). However, elevated levels of *Blautia* were reported in relation to MDD in adults (Cheung et al., 2019) and ADHD in three-year-old children (Laue et al., 2021), indicating that different mechanisms may be involved depending on the psychopathology and chronological age. Lastly, we observed a positive relation between one unidentified *Enterobacteriaceae* genus at the age of two weeks and externalizing problems at the age of three years. Similarly, more *Enterobacteriaceae* species were cross-sectionally related to decreased cognitive functioning at the age of 45 months (Streit et al., 2021).

Another of our findings was that higher alpha diversity at age two weeks was linked to fewer internalizing problems and better executive functions at age three years. In accordance with our internalizing behavior result, Laue et al. observed that higher alpha diversity in the first two months of life was related to less internalizing behavior in three-year-old boys (Laue et al., 2021). Furthermore, van de Wouw et al. found lower alpha diversity in clinically diagnosed internalizing behavior (Van De Wouw et al., 2022). This may indicate that higher alpha diversity is related to improved subsequent mental outcomes in toddlerhood. On the contrary, Carlson et al. found higher alpha diversity at age one year related to worse cognition at age two years in typically developing toddlers (Carlson et al., 2017). Speculatively, higher alpha diversity in the first postnatal weeks has a different meaning than higher alpha diversity at age one year, and this may explain the opposite findings by Carlson et al. Although underlying mechanisms behind the described links have not yet been elaborated, critical molecular pathways might involve neurotransmitters such as GABA (Altaib et al., 2021) and norepinephrine (mainly targeted at digestive system) (Barandouzi et al., 2022).

Our study contributes to the growing body of literature on the gut microbiota, problem behavior, and executive functions. A strength of our study is the longitudinal design, which covered the period from birth to age three years and allowed for the assessment of multiple developmental stages of the gut microbiota. Another advantage was that questionnaires were filled in by both mother and partner. Partner reports were used for sensitivity analyses and because they showed positive correlations with maternal reports, they enhanced the reliability of our study measures. Furthermore, problem behavior and executive functions were assessed with multiple questionnaires (i.e., CBCL and SDQ for problem behavior, and BRIEF-P and REEF for executive functions), allowing us to determine conformity and consistency between various questionnaires. Finally, we used standardized behavioral tasks as a tool to objectively determine child executive functions.

A limitation of our study is the possible overreliance on the compositional features of the gut microbiota using relative abundances instead of absolute abundances. This

approach may increase the chance of spurious associations as relative abundances are dependent on each other. Besides, 16S rRNA sequence data are limited at species-level resolution and profiling precise gene functions (Durazzi et al., 2021). Suggested potential underlying mechanisms regarding neurotransmitters need to be carefully addressed in follow-up studies, preferably preclinical experimental studies. Taken together, further applications, such as quantitative PCR, whole-genome shotgun metagenomic sequencing, targeted fecal metabolomics, and experimental studies in animal models, would improve the understanding of current correlational results and provide insight into causality. Another limitation of our study is the relatively small sample size and mostly highly educated study population, limiting the generalizability of the findings. The restricted sample size may also hamper deep inference with respect to taxa with low prevalence rates to some degree. Our findings on microbial relations to the mental outcomes and speculations on underlying mechanisms of these relations need to be confirmed in a larger, more representative cohort. Lastly, in addition to including microbial taxon abundances as continuous variables, transforming abundance data into binary variables (absence and presence) may cast more light on a panoramic view of such relations.

To conclude, our results provide tentative evidence supporting the idea that in a child's first years of life the gut microbiota might play a vital role in the development of the brain. Potential mechanisms are likely related to microbiota-derived metabolites (Ahmed et al., 2022). As the nature of this study was exploratory and the body of similar research needs to grow to a large extent, it is still premature to translate our correlational findings into clinical implications. Replications in other longitudinal studies on healthy community children are necessary to confirm our findings and to shed more light on key microbial taxa and latent pathways of associations between early gut microbiota and child behavior and cognition.

Data availability statement

BINGO data sets including metadata and sequence data currently cannot be made publicly available due to the data being part of an ongoing longitudinal study. The data are available upon request to Prof. Dr. C. de Weerth at Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, the Netherlands (e-mail: Carolina.deWeerth@radboudumc.nl).

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Supplemental information

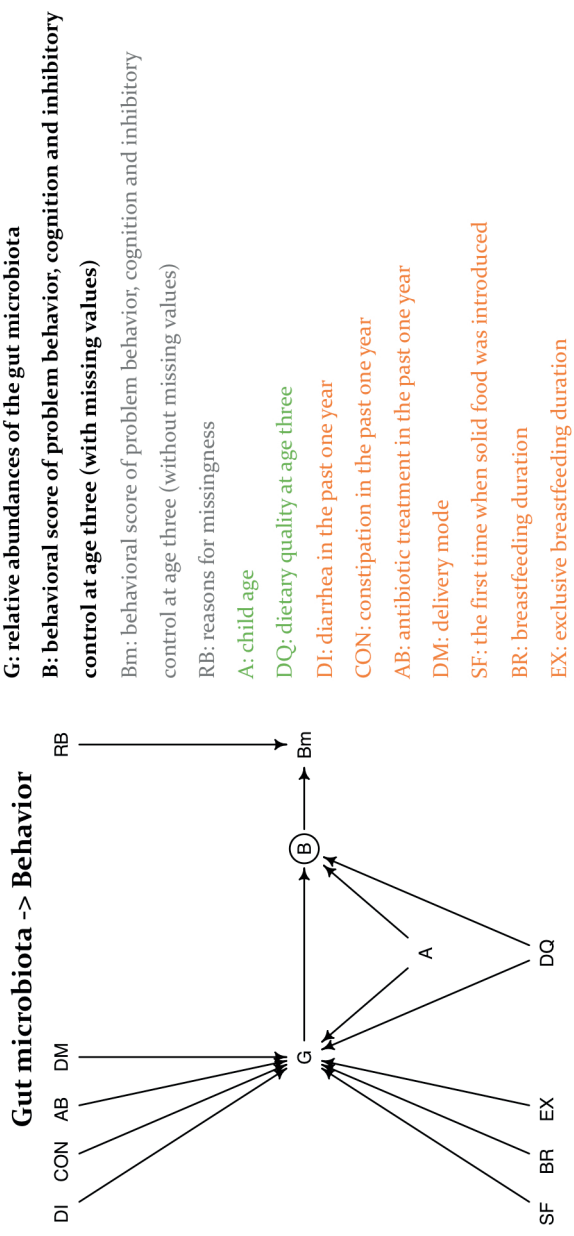

Figure S1. Directed Acyclic Graph for determining confounders.

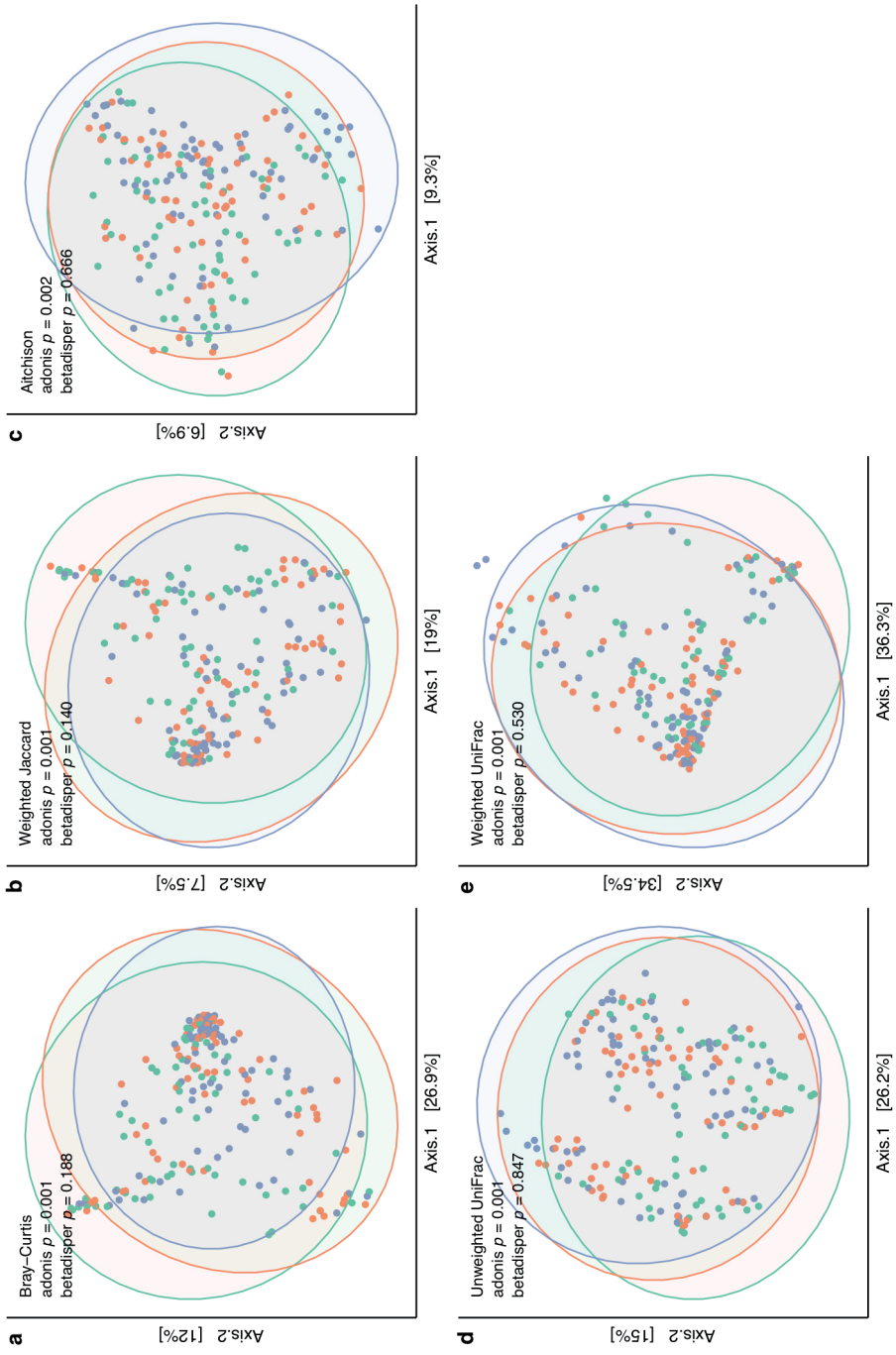

Figure S2. Beta diversity of the gut microbiota at the age of two, six, and 12 weeks. (a-e) Principal coordinate plots of beta diversity, based on different pairwise dissimilarity (Bray-Curtis and weighted Jaccard) and distance (UniFrac and Aitchison) matrices, with points and ellipses colored by ages (Lake blue, two weeks; Orange, six weeks; Purple, 12 weeks).

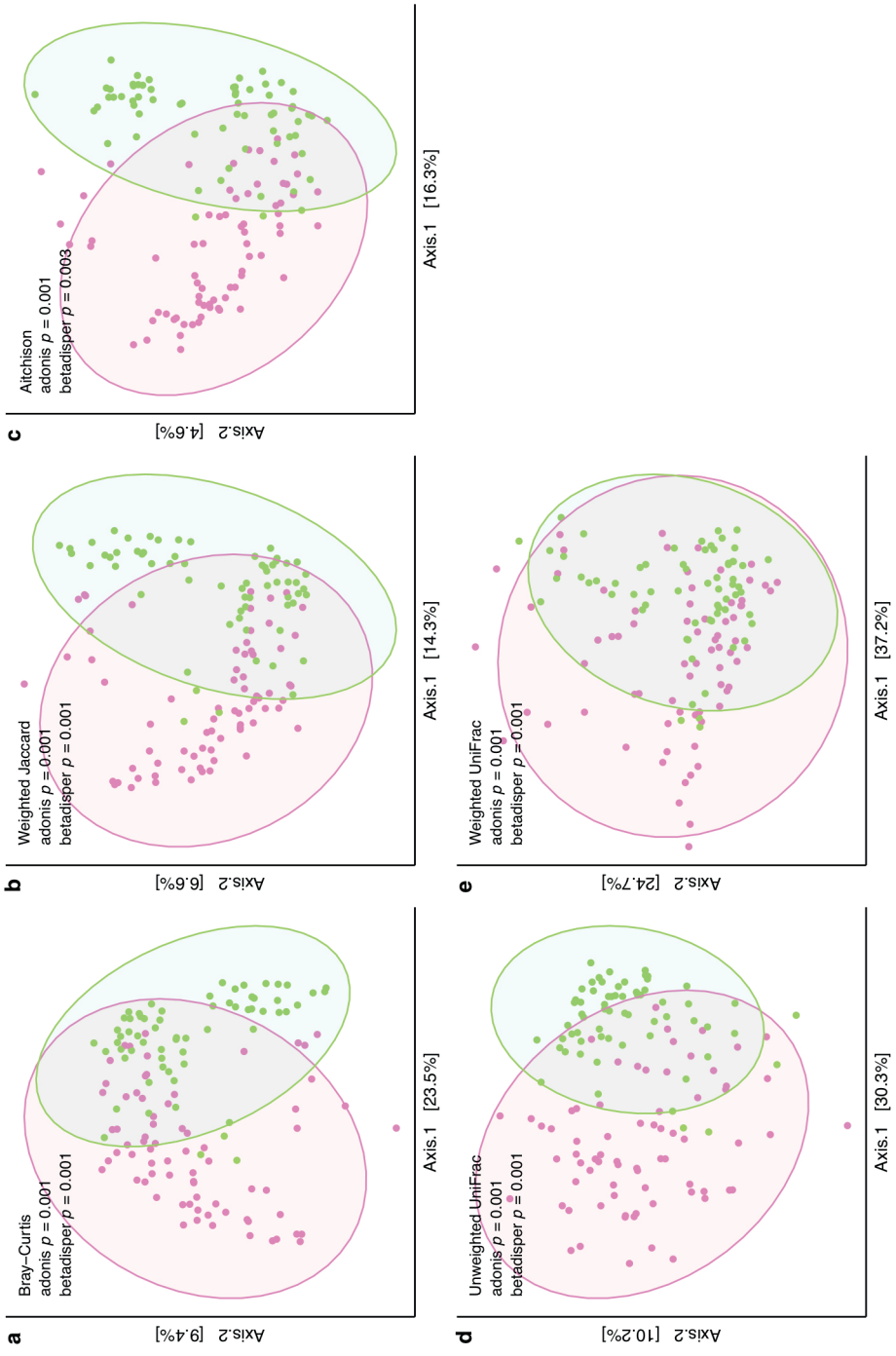

Figure S3. Beta diversity of the gut microbiota at the age of one and three years. (a-e) Principal coordinate plots of beta diversity, based on different pairwise dissimilarity (Bray-Curtis and weighted Jaccard) and distance (UniFrac and Aitchison) matrices, with points and ellipses colored by ages (Pink, one year; Grass green, three years).

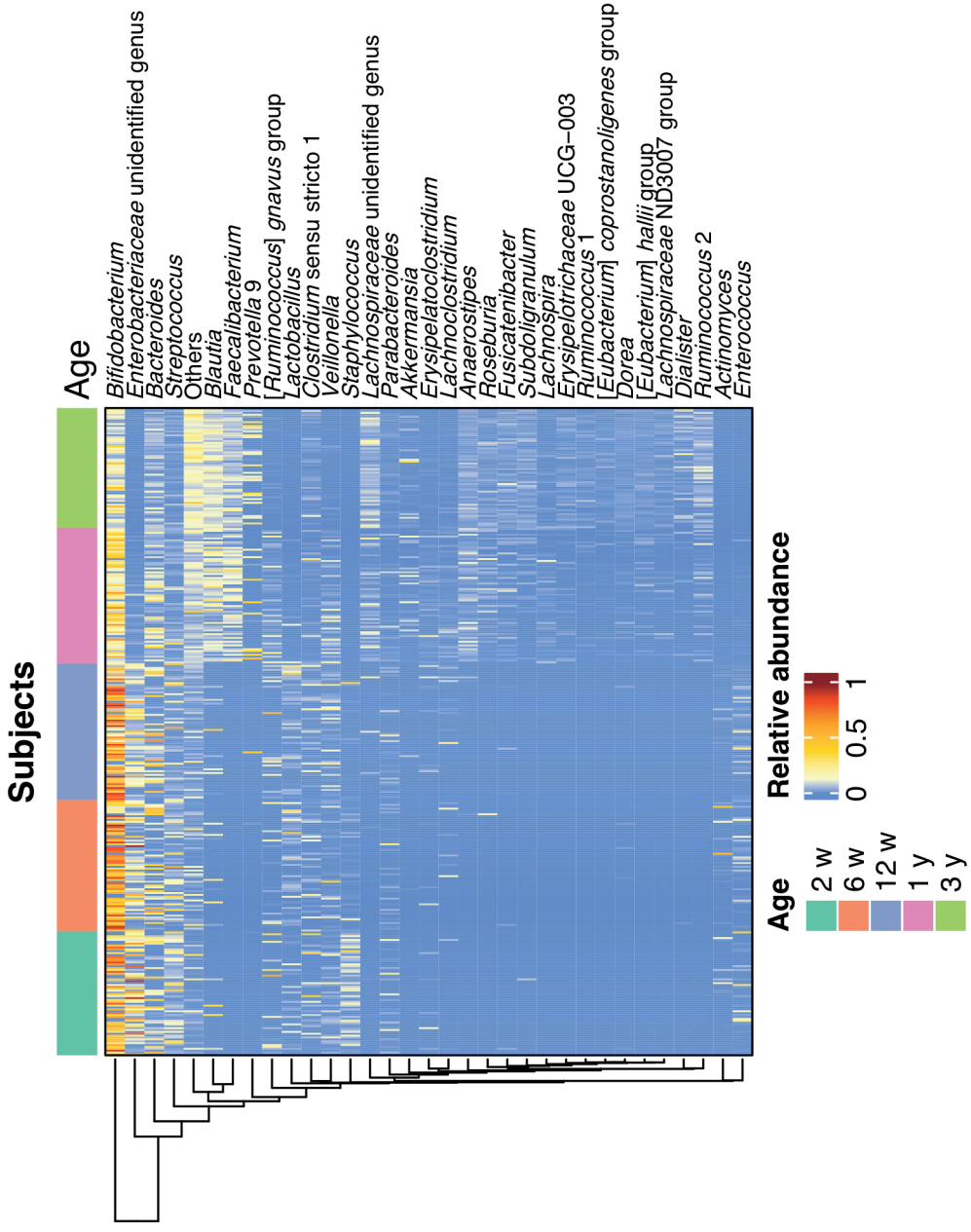

Figure S4. Heatmap showing relative abundances of the gut microbiota at the genus level over time. Bacteria with average relative abundances lower than 1% across the first three years, were assigned to 'Others'. Rows of bacteria were clustered based on Euclidean distance.

Table S1. Kendall correlations between behavioral measures.

	CBCL_M_Internalizing	CBCL_M_Externalizing	CBCL_P_Internalizing	CBCL_P_Externalizing	SDQ_M_Internalizing	SDQ_M_Externalizing	SDQ_P_Internalizing	SDQ_P_Externalizing	BRIEF-P_M_TotalScore	BRIEF-P_P_TotalScore	REEF_M_TotalScore	REEF_P_TotalScore	Flanker	GiftWrap	GiftDelay	Whisper
CBCL_M_Internalizing	-															
CBCL_M_Externalizing	0.49*	-														
CBCL_P_Internalizing	0.4*	0.21	-													
CBCL_P_Externalizing	0.32*	0.29*	0.54*	-												
SDQ_M_Internalizing	0.4*	0.12	0.21	0.14	-											
SDQ_M_Externalizing	0.14	0.39*	-0.02	0.22	0	-										
SDQ_P_Internalizing	0.3*	0.17	0.34*	0.24	0.31*	-0.06	-									
SDQ_P_Externalizing	0.11	0.26	0.16	0.35*	0.15	0.32*	0.18	-								
BRIEF-P_M_TotalScore	0.44*	0.53*	0.27	0.24	0.2	0.29*	0.18	0.22	-							

BRIEF-P_P_TotalScore	0.32*	0.21	0.38*	0.37*	0.27	0.21	0.38*	0.23	0.39*	-0.21						
REEF_M_TotalScore	-0.19	-0.27*	-0.21	-0.18	-0.1	-0.16	-0.09	0.03	-0.34*	-0.21	-0.24					
REEF_P_TotalScore	0.02	-0.19	-0.03	-0.23	-0.01	-0.34*	-0.09	-0.25	-0.13	-0.13	0.24	-0.04				
Flanker	-0.01	0.02	-0.18	-0.02	0.08	0.01	0.04	0.09	0.01	-0.16	0.07	0.04	-0.05			
GiftWrap	-0.07	-0.03	0.06	0.05	0	-0.09	-0.09	0.17	0.02	0.01	0.15	0.1	0.05	-0.11		
GiftDelay	0.06	0.02	0.15	0.29*	0.14	-0.17	0.22	0.2	0.01	0	0.18	0.07	0.19	0.04	-0.25*	
Whisper	-0.06	-0.04	-0.1	-0.09	-0.01	-0.1	-0.21	-0.21	-0.14	-0.23	0.21	0.19	0.11	0.04	0.25*	-0.11

M, Mother; P, Partner; CBCL, the Child Behavioral Checklist; SDQ, the Strengths and Difficulties Questionnaire; BRIEF-P, Behavior Rating Inventory of Executive Functions - Preschool; REEF, Ratings of Everyday Executive Functioning. Note that increased internalizing and externalizing scores refer to more corresponding behavioral problems. A higher score on the BRIEF-P indicates worse executive functions while a higher score on the REEF indicates better executive functions. Higher scores on the four behavioral tasks mean better performances in inhibitory control. * indicates a p value lower than 0.05.

Table S2. Reliability of parental questionnaires.

Filler	Questionnaire	behavior	ω_{total}	Cronbach's α
Mother	CBCL	Internalizing	IC	0.83
		Externalizing	0.92	-
	SDQ	Internalizing	0.65	-
		Externalizing	0.74	-
	BRIEF-P	Executive functions	0.94	-
	REEF	Executive functions	IC	0.96
Partner	CBCL	Internalizing	IC	0.83
		Externalizing	0.84	-
	SDQ	Internalizing	0.65	-
		Externalizing	0.72	-
	BRIEF-P	Executive functions	IC	0.95
	REEF	Executive functions	IC	0.95

IC indicates the estimate was incalculable. Due to better ability at assessing reliability, ω_{total} values were used as the first important estimates in determining reliability. For those subscales and questionnaires with incalculable ω_{total}, Cronbach's α values were computed as alternatives. All estimates were above 0.65, of which most of estimates were higher than 0.7, indicating good reliability of the scales.

Table S3. Proportion of missing values in problem behavior and executive functions.

	Proportion of missing values				
	2w	6w	12w	1y	3y
CBCL_M_Internalizing	16.7%	14.3%	13.7%	12.5%	3.1%
CBCL_M_Externalizing	16.7%	14.3%	13.7%	12.5%	3.1%
SDQ_M_Internalizing	16.7%	14.3%	13.7%	12.5%	3.1%
SDQ_M_Externalizing	16.7%	14.3%	13.7%	12.5%	3.1%
BRIEF-P_M_TotalScore	15.2%	12.9%	12.3%	11.1%	1.6%
REEF_M_TotalScore	16.7%	14.3%	13.7%	12.5%	3.1%
Flanker	40.9%	35.7%	37%	34.7%	26.6%
Whisper	19.7%	17.1%	17.8%	15.3%	6.2%
Gift Wrap	21.2%	20%	19.2%	16.7%	6.2%
Gift Delay	19.7%	18.6%	17.8%	15.3%	4.7%

Notes. M, Mother; CBCL, the Child Behavioral Checklist; SDQ, the Strengths and Difficulties Questionnaire; BRIEF-P, Behavior Rating Inventory of Executive Functions - Preschool; REEF, Ratings of Everyday Executive Functioning.

Table S4. Differentially abundant microbial taxa at the genus level over time with linear discriminant analysis (LDA) scores higher than 2.

Enriched age	Genus	LDA score
2 w	*Enterobacteriaceae* unidentified genus	4.93
2 w	*Streptococcus*	4.56
2 w	*Staphylococcus*	4.43
2 w	[*Ruminococcus*] *gnavus* group	4.3
2 w	*Enterococcus*	4.1
2 w	*Clostridium* sensu stricto 1	3.99
2 w	*Parabacteroides*	3.84
2 w	*Finegoldia*	2.49
2 w	*Negativicoccus*	2.3
6 w	*Lactobacillus*	4.31
6 w	*Actinomyces*	3.99
6 w	*Hungatella*	3.42
6 w	*Megasphaera*	3.4
6 w	*Candidatus Stoquefichus*	2.5
6 w	*Halomonas*	2.49
6 w	*Aeribacillus*	2.12
12 w	*Bifidobacterium*	5.3
12 w	*Bacteroides*	4.54
12 w	*Rothia*	2.85
12 w	*Varibaculum*	2.85
12 w	*Ruminiclostridium*	2.15
1 y	*Faecalibacterium*	4.69
1 y	*Anaerostipes*	4.32
1 y	*Veillonella*	4.23
1 y	*Akkermansia*	4.01
1 y	*Lachnoclostridium*	3.79
1 y	*Erysipelatoclostridium*	3.77
1 y	*Lachnospira*	3.75
1 y	[*Eubacterium*] *eligens* group	3.55
1 y	*Prevotella* 2	3.38
1 y	[*Clostridium*] *innocuum* group	3.38
1 y	*Flavonifractor*	3.2
1 y	*Sutterella*	3.16
1 y	*Tyzzerella* 4	3.09
1 y	*Lachnospiraceae* UCG-004	3.08
1 y	*Eggerthella*	2.98
1 y	*Parasutterella*	2.97
1 y	*Clostridioides*	2.67
1 y	*Tyzzerella* 3	2.6
1 y	CAG:352	2.54
1 y	*Lactococcus*	2.48
3 y	*Blautia*	4.78
3 y	*Prevotella* 9	4.56
3 y	*Ruminococcus* 2	4.31
3 y	*Fusicatenibacter*	4.09
3 y	*Roseburia*	4.08
3 y	*Subdoligranulum*	4.06

3 y	*Dialister*	4.03
3 y	[*Eubacterium*] *hallii* group	3.88
3 y	*Erysipelotrichaceae* UCG-003	3.87
3 y	*Dorea*	3.85
3 y	*Lachnospiraceae* ND3007 group	3.79
3 y	*Ruminococcus* 1	3.74
3 y	*Lachnospiraceae* NK4A136 group	3.65
3 y	*Intestinibacter*	3.65
3 y	[*Eubacterium*] *coprostanoligenes* group	3.65
3 y	[*Ruminococcus*] *torques* group	3.58
3 y	*Ruminococcaceae* UCG-002	3.53
3 y	*Alistipes*	3.5
3 y	uncultured genus	3.49
3 y	*Christensenellaceae* R-7 group	3.45
3 y	*Romboutsia*	3.42
3 y	*Phascolarctobacterium*	3.41
3 y	*Butyricicoccus*	3.4
3 y	*Coprococcus* 2	3.4
3 y	uncultured bacterium	3.35
3 y	[*Ruminococcus*] *gauvreauii* group	3.33
3 y	*Barnesiella*	3.26
3 y	*Coprococcus* 3	3.25
3 y	*Prevotella* 7	3.2
3 y	*Senegalimassilia*	3.14
3 y	*Coprococcus* 1	3.06
3 y	*Ruminococcaceae* UCG-013	3.02
3 y	*Holdemanella*	2.99
3 y	*Paraprevotella*	2.99
3 y	*Terrisporobacter*	2.98
3 y	[*Eubacterium*] *ventriosum* group	2.98
3 y	*Ruminococcaceae* NK4A214 group	2.94
3 y	*Sarcina*	2.93
3 y	*Ruminococcaceae* UCG-005	2.93
3 y	*Sellimonas*	2.9
3 y	[*Eubacterium*] *ruminantium* group	2.81
3 y	*Lachnospiraceae* UCG-001	2.81
3 y	*Ruminiclostridium* 6	2.8
3 y	*Lachnospiraceae* FCS020 group	2.78
3 y	*Ruminococcaceae* UCG-014	2.76
3 y	*Ruminococcaceae* UCG-004	2.75
3 y	*Adlercreutzia*	2.67
3 y	*Lachnospiraceae* UCG-003	2.64
3 y	CAG:56	2.63
3 y	[*Eubacterium*] *xylanophilum* group	2.63
3 y	*Alloprevotella*	2.62
3 y	*Gordonibacter*	2.54
3 y	*Ruminiclostridium* 5	2.53
3 y	*Ruminococcaceae* UCG-003	2.52
3 y	*Turicibacter*	2.51
3 y	*Butyrivibrio*	2.5
3 y	Family XIII AD3011 group	2.45
3 y	*Odoribacter*	2.4

3 y	*Mollicutes* RF39 uncultured bacterium	2.4
3 y	*Oscillibacter*	2.36
3 y	*Anaeroplasma*	2.23
3 y	*Methanobrevibacter*	2.22
3 y	*Marvinbryantia*	2.15
3 y	*Butyricimonas*	2.1
3 y	*Ruminiclostridium* 9	2.09

Table S5. Pearson correlations between actual and predicted results from random forest models.

Behavior at age three	Age of the gut microbiota	Median of Pearson correlation coefficient	Permutation *p* value	Adjusted permutation *p* value
CBCL_M_Internalizing	2w	0.04	0.91	1.00
	6w	0.05	0.87	1.00
	12w	0.02	0.96	1.00
	1y	-0.09	0.80	1.00
	3y	-0.16	0.59	1.00
CBCL_M_Externalizing	2w	0.00	0.99	1.00
	6w	-0.33	0.32	1.00
	12w	0.13	0.71	1.00
	1y	0.09	0.78	1.00
	3y	-0.11	0.73	1.00
SDQ_M_Internalizing	2w	0.05	0.88	1.00
	6w	-0.03	0.91	1.00
	12w	0.28	0.37	1.00
	1y	-0.16	0.67	1.00
	3y	0.06	0.85	1.00
SDQ_M_Externalizing	2w	0.05	0.88	1.00
	6w	-0.29	0.37	1.00
	12w	0.10	0.78	1.00
	1y	0.02	0.94	1.00
	3y	0.14	0.67	1.00
BRIEF-P_M_TotalScore	2w	0.12	0.70	1.00
	6w	0.07	0.85	1.00
	12w	0.10	0.77	1.00
	1y	0.06	0.82	1.00
	3y	-0.09	0.76	1.00
REEF_M_TotalScore	2w	-0.15	0.65	1.00
	6w	-0.07	0.84	1.00
	12w	-0.15	0.66	1.00
	1y	-0.01	0.99	1.00
	3y	-0.11	0.74	1.00
Flanker	2w	-0.07	0.83	1.00
	6w	0.32	0.32	1.00
	12w	0.07	0.84	1.00
	1y	-0.17	0.63	1.00
	3y	0.07	0.83	1.00
Whisper	2w	0.09	0.82	1.00
	6w	-0.06	0.88	1.00
	12w	0.23	0.52	1.00
	1y	0.28	0.37	1.00
	3y	-0.03	0.93	1.00
GiftWrap	2w	0.02	0.94	1.00
	6w	0.25	0.39	1.00
	12w	0.14	0.67	1.00
	1y	-0.02	0.93	1.00
	3y	-0.37	0.23	1.00

GiftDelay	2w	-0.15	0.56	1.00
	6w	-0.09	0.89	1.00
	12w	0.00	1.00	1.00
	1y	0.32	0.32	1.00
	3y	0.04	0.91	1.00

N=1000 permutation tests were performed.
FDR adjustments were conducted to the p values.

Table S6. Microbial taxa and alpha diversity with confident age-stratified relations to behavioral measures and taxa prevalence over time.

Type	Behavior at age three	Taxa or alpha diversity	Prevalence at 2w	Prevalence at 6w	Prevalence at 12w	Prevalence at 1y	Prevalence at 3y	Prevalence >10% at all ages	Prevalence >10% only at the first three ages	Prevalence >10% only at the last two ages
genus	CBCL_Internalizing	*Barnesiella*	0	1	0	7	50	no	no	no
genus	CBCL_Internalizing	*Intestinibacter*	0	9	16	78	88	no	no	no
genus	CBCL_Externalizing	*Barnesiella*	0	1	0	7	50	no	no	no
genus	CBCL_Externalizing	*Butyricicoccus*	0	0	3	56	89	no	no	yes
genus	CBCL_Externalizing	*Clostridium* sensu stricto 1	33	50	44	62	83	yes	no	no
genus	CBCL_Externalizing	*Parabacteroides*	35	34	36	44	83	yes	no	no
genus	CBCL_Externalizing	*Streptococcus*	94	93	88	90	88	yes	no	no
genus	SDQ_Internalizing	[*Ruminococcus*] *torques* group	5	6	4	32	84	no	no	yes
genus	SDQ_Internalizing	*Bifidobacterium*	79	87	93	99	100	yes	no	no
genus	SDQ_Internalizing	*Blautia*	6	11	14	90	100	no	no	no
genus	SDQ_Internalizing	*Ruminococcus* 2	0	0	0	39	94	no	no	yes
genus	SDQ_Internalizing	*Sutterella*	8	10	12	46	61	no	no	no
genus	SDQ_Externalizing	*Bifidobacterium*	79	87	93	99	100	yes	no	no

genus	SDQ_Externalizing	*Butyricicoccus*	0	0	3	56	89	no	no	yes
genus	SDQ_Externalizing	*Enterobacteriaceae* unidentified genus	89	93	97	68	31	yes	no	no
genus	SDQ_Externalizing	*Halomonas*	12	11	12	0	0	no	yes	no
genus	SDQ_Externalizing	*Oscillibacter*	0	0	0	4	22	no	no	no
genus	SDQ_Externalizing	*Parabacteroides*	35	34	36	44	83	yes	no	no
genus	BRIEF-P	*Blautia*	6	11	14	90	100	no	no	no
genus	BRIEF-P	*Clostridium* sensu stricto 1	33	50	44	62	83	yes	no	no
genus	BRIEF-P	*Halomonas*	12	11	12	0	0	no	yes	no
genus	BRIEF-P	*Intestinibacter*	0	9	16	78	88	no	no	no
genus	BRIEF-P	*Ruminococcus* 2	0	0	0	39	94	no	no	yes
genus	BRIEF-P	*Streptococcus*	94	93	88	90	88	yes	no	no
genus	REEF	[*Ruminococcus*] *torques* group	5	6	4	32	84	no	no	yes
genus	REEF	*Halomonas*	12	11	12	0	0	no	yes	no
genus	REEF	*Lachnospiraceae* unidentified genus	5	4	15	78	100	no	no	no
genus	REEF	*Parabacteroides*	35	34	36	44	83	yes	no	no
genus	Flanker	*Anaerostipes*	0	1	3	96	100	no	no	yes
genus	Flanker	*Bacteroides*	56	59	66	86	97	yes	no	no
genus	Flanker	*Ruminococcaceae* UCG-013	0	0	0	33	73	no	no	yes
genus	Flanker	*Subdoligranulum*	2	0	0	31	95	no	no	yes
genus	Flanker	*Sutterella*	8	10	12	46	61	no	no	no

genus	GiftWrap	*Coprococcus 3*	0	0	0	14	66	no	no	yes
genus	GiftWrap	*Lachnospiraceae* NK4A136 group	0	0	0	38	89	no	no	yes
genus	GiftWrap	*Subdoligranulum*	2	0	0	31	95	no	no	yes
genus	GiftWrap	*Veillonella*	62	64	62	71	17	yes	no	no
alpha diversity	CBCL_Internalizing	cha01	-	-	-	-	-	-	-	-
alpha diversity	REEF	cha01	-	-	-	-	-	-	-	-
alpha diversity	GiftWrap	cha01	-	-	-	-	-	-	-	-
alpha diversity	GiftWrap	PD	-	-	-	-	-	-	-	-

Table S7. The multilevel Bayesian results of selected genera and alpha diversity with behavioral measures.

Behavior at age three	Taxa or alpha diversity	Age of the gut microbiota	Rhat <1.01	Estimate	Estimate error	95% CI	95% CI excluding 0
CBCL_Externalizing	*Clostridium* sensu stricto 1	2w, 6w, 12w, 1y, 3y	yes	0	0	[-0.01, 0.01]	no
CBCL_Externalizing	*Parabacteroides*	2w, 6w, 12w, 1y, 3y	no	-	-	-	-
CBCL_Externalizing	*Streptococcus*	2w, 6w, 12w, 1y, 3y	yes	0.03	0.02	[0, 0.07]	no
SDQ_Internalizing	*Bifidobacterium*	2w, 6w, 12w, 1y, 3y	yes	0.09	0.07	[-0.04, 0.22]	no
SDQ_Externalizing	*Bifidobacterium*	2w, 6w, 12w, 1y, 3y	yes	-0.04	0.07	[-0.17, 0.09]	no
SDQ_Externalizing	*Enterobacteriaceae* unidentified genus	2w, 6w, 12w, 1y, 3y	yes	0.01	0.01	[-0.01, 0.03]	no
SDQ_Externalizing	*Parabacteroides*	2w, 6w, 12w, 1y, 3y	no	-	-	-	-
BRIEF-P	*Clostridium* sensu stricto 1	2w, 6w, 12w, 1y, 3y	yes	0	0	[0, 0.01]	no
BRIEF-P	*Streptococcus*	2w, 6w, 12w, 1y, 3y	yes	0.05	0.02	[0.02, 0.09]	yes
REEF	*Parabacteroides*	2w, 6w, 12w, 1y, 3y	no	-	-	-	-
Flanker	*Bacteroides*	2w, 6w, 12w, 1y, 3y	no	-	-	-	-
GiftWrap	*Veillonella*	2w, 6w, 12w, 1y, 3y	yes	0.01	0	[0, 0.02]	no
CBCL_Internalizing	chao1	2w, 6w, 12w, 1y, 3y	yes	-0.01	0.02	[-0.06, 0.04]	no
REEF	chao1	2w, 6w, 12w, 1y, 3y	yes	0.04	0.03	[-0.01, 0.09]	no
GiftWrap	chao1	2w, 6w, 12w, 1y, 3y	yes	0.02	0.03	[-0.03, 0.07]	no
GiftWrap	PD	2w, 6w, 12w, 1y, 3y	yes	0.05	0.03	[-0.01, 0.12]	no
CBCL_Externalizing	*Parabacteroides*	2w, 6w, 12w	no	-	-	-	-
SDQ_Externalizing	*Halomonas*	2w, 6w, 12w	no	-	-	-	-
SDQ_Externalizing	*Parabacteroides*	2w, 6w, 12w	no	-	-	-	-
BRIEF-P	*Halomonas*	2w, 6w, 12w	no	-	-	-	-
REEF	*Halomonas*	2w, 6w, 12w	no	-	-	-	-
REEF	*Parabacteroides*	2w, 6w, 12w	no	-	-	-	-
Flanker	*Bacteroides*	2w, 6w, 12w	yes	0.01	0.01	[-0.01, 0.03]	no
CBCL_Externalizing	*Butyricicoccus*	1y, 3y	yes	-0.01	0.08	[-0.17, 0.15]	no

CBCL_Externalizing	*Parabacteroides*	1y, 3y	no	-	-	-	-
SDQ_Internalizing	[*Ruminococcus*] *torques* group	1y, 3y	yes	-0.22	0.07	[-0.35, -0.07]	yes
SDQ_Internalizing	*Ruminococcus* 2	1y, 3y	yes	-0.1	0.08	[-0.26, 0.05]	no
SDQ_Externalizing	*Butyricicoccus*	1y, 3y	yes	-0.09	0.09	[-0.25, 0.08]	no
SDQ_Externalizing	*Parabacteroides*	1y, 3y	yes	-0.04	0.03	[-0.09, 0.02]	no
BRIEF-P	*Ruminococcus* 2	1y, 3y	yes	-0.11	0.08	[-0.25, 0.04]	no
REEF	[*Ruminococcus*] *torques* group	1y, 3y	yes	-0.05	0.08	[-0.21, 0.09]	no
REEF	*Parabacteroides*	1y, 3y	yes	-0.01	0.03	[-0.06, 0.05]	no
Flanker	*Anaerostipes*	1y, 3y	yes	-0.05	0.09	[-0.23, 0.11]	no
Flanker	*Bacteroides*	1y, 3y	yes	-0.07	0.08	[-0.23, 0.08]	no
Flanker	*Ruminococcaceae* UCG-013	1y, 3y	yes	0.1	0.1	[-0.09, 0.29]	no
Flanker	*Subdoligranulum*	1y, 3y	no	-	-	-	-
GiftWrap	*Coprococcus* 3	1y, 3y	no	-	-	-	-
GiftWrap	*Lachnospiraceae* NK4A136 group	1y, 3y	yes	-0.07	0.05	[-0.17, 0.01]	no
GiftWrap	*Subdoligranulum*	1y, 3y	yes	-0.03	0.06	[-0.16, 0.06]	no

Notes. Multilevel Bayesian linear regression models were performed on taxa and alpha diversity over time. Relations in gray rows are confident with 95% CI excluding zero. Chains were regarded converged when Rhat values lower than 1.01. Models that did not meet the Rhat criteria under current settings were not considered in the present study. Given that *Parabacteroides* and *Bacteroides* did not meet the Rhat requirement in the pooled data of all five ages, we did extra trajectory analyses for them after splitting the data into two periods (2w, 6w, and 12w; 1y and 3y; colored in gray).

Table S8. Overview of the associations in our study in comparison with findings reported in literature.

Microbial taxa and alpha diversity	In the present study	Literature about problem behavior or executive functions and inhibitory control	Other literature with similar findings	Other literature with divergent findings	Potential mechanisms
↑*Parabacteroides* (2w)	↓ Externalizing behavior	NF		↑*Parabacteroides* in Children with ASD (Coretti et al., 2018; Inoue et al., 2016; Plaza-Díaz et al., 2019)	GABA
	↑ Executive functions	NF	↓*Parabacteroides* in ADHD (Prehn-Kristensen et al., 2018)		
↑*Parabacteroides* (1y)	↓ Externalizing behavior	NF			
↑*Ruminococcus* 2 (1y)	↓ Internalizing behavior	NF	↓*Ruminococcus* 2 in MDD patients (Cheung et al., 2019; Haiyin Jiang et al., 2015)		Tryptophan / serotonin
	↑ Executive functions	NF		↑*Ruminococcaceae* in ADHD patients, inattention (Szopinska-Tokov et al., 2020)	
↑ [*Ruminococcus*] *Torques* group (3y)	↓ Executive functions	NF			
↑*Barnesiella* (3y)	↑ Internalizing and externalizing behavior	NF	↑*Barnesiella* in (constipated) ASD (S. Liu et al., 2019) (Zhao et al., 2019)	↓*Barnesiella* in ASD (Averina et al., 2020)	GABA

↑*Butyricicoccus* (1y)	↓ Externalizing behavior	NF	↑*Butyricicoccus* in constipated ASD vs non-constipated ASD (Dan et al., 2020)	Butyrate
↑*Butyricicoccus* (3y)	↑ Externalizing behavior	NF	↓*Butyricicoccus* in ASD (S. Liu et al., 2019)	
↑*Streptococcus* (2w)	↑ Externalizing behavior	NF	↑*Streptococcus* ASD (Bundgaard-Nielsen et al., 2020); ↑*Streptococcus* in Bipolar disorder (Järbrink-Sehgal & Andreasson, 2020)	GABA and tryptophan
	↓ Executive functions	NF		
↑*Streptococcus* (12w)	↓ Executive functions	NF		
↑*Clostridium* sensu stricto 1 (1y)	↑ Externalizing behavior		↑*Clostridium* in ASD (De Angelis et al., 2013; Kandeel et al., 2020)	Neurotoxins
	↓ Executive functions	↑*Clostridium* at 2.5 months with attention (Aatsinki et al., 2020)		
↑*Intestinibacter* (12w)	↑ Internalizing behavior	NF	↑*Intestinibacter* bartlettii more in children with neurodevelopmental disorders (Bojović et al., 2020)	Neurotoxins.
	↓ Executive functions	NF		

↑*Bifidobacterium* (3y)	↑ Internalizing behavior	NF	↑*Bifidobacterium* in MDD patients (Knudsen et al., 2021)	↓*Bifidobacterium* in ASD (Xu et al., 2019); ↑*Bifidobacterium* less ASD symptoms (Grimaldi et al., 2017); ↓*Bifidobacterium* in MDD patients (Cheung et al., 2019)	GABA, dopamine and noradrenaline
	↑ Externalizing behavior	NF	↑*Bifidobacterium* in ADHD patients (Aarts et al., 2017)	↑*Bifidobacterium longus* positive on ADHD (Finegold et al., 2010; Pärtty et al., 2015)	
↑*Blautia* (3y)	↓ Internalizing behavior	NF	↓*Blautia* in ASD patients (F. Liu et al., 2019)	↑*Blautia* in MDD (Cheung et al., 2019)	NF
	↑ Executive functions	NF		↑*Blautia* with worse ADHD symptoms (Laue et al., 2021)	
↑*Halomonas* (6w)	↑ Externalizing behavior	NF			GABA and tryptophan
	↓ Executive functions	NF	↑*Halomonas* in Alzheimers (Ling et al., 2021)		
	↑ Inhibitory control	↑ *Bacteroides* with better cognition at 2 years (Carlson et al., 2017; Tamana et al., 2021)			
↑ *Bacteroides* (6w)					GABA
↑ *Subdoligranulum* (1 y and 3y)	↓ Inhibitory control	NF	↑ *Subdoligranulum* in patients with anxiety		NF

			(Chen et al., 2019)		
			↓ *Anaerostipes* in children with autism (Iglesias-Vásquez et al., 2022)		
↑ *Anaerostipes* (1 y)	↓ Inhibitory control	NF			Butyrate
↑ *Lachnospiraceae* NK4A136 (1y)	↓ Inhibitory control	NF			NF
↑ *Ruminococcaceae* UCG-013 (1y)	↑ Inhibitory control	NF			NF
↑*Sutterella* (1y)	↓ Inhibitory control	↑ *Sutterella* with better cognition at age three years (Rothenberg et al., 2021)	↑ *Sutterella* in children with autism (L. Wang et al., 2013; B. L. Williams et al., 2012)		NF
				↑ *Coprococcus* 3 in healthy patients compared to patients with anxiety disorder (Chen et al., 2019)	
↑ *Coprococcus* 3 (1y)	↓ Inhibitory control	NF			Tryptophan Immune system, interleukin pathways
↑ *Veillonella* (1y)	↑ Inhibitory control	↑ *Veillonella* with better cognition at five years (Guzzardi et al., 2022)			
↑Alpha diversity (2w)	↓Internalizing behavior	↑alpha diversity with less internalizing behavior in boys Laue et	↓alpha diversity in ASD children (Chen et al., 2021; Kang et al., 2018; S. Liu et al., 2019; Ma et al., 2019)		GABA and norepinephrine

	al. (2021); ↓Alpha diversity in children above the clinical threshold for internalizing behavior (van de Wouw et al., 2021)		
↑Executive functions	↑alpha diversity and worse cognition (Carlson et al. 2017)	↓alpha diversity in ADHD (Prehn-Kristensen et al., 2018)	No differences in alpha diversity between ADHD patients and healthy controls (Hai yin Jiang et al., 2018; Richarte et al., 2021; Szopinska-Tokov et al., 2020; Wan et al., 2020).

Notes. NF, Not Found (i.e., no comparable findings in the literature for behavioral problems or executive functions).

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Chapter 3

Development of the gut microbiota in healthy children in the first ten years of life: associations with internalizing and externalizing behavior

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Abstract

Background: Increasing evidence indicates that psychopathological disorders are associated with the gut microbiota. However, data is largely lacking from long-term longitudinal birth cohorts, especially those comprising low-risk healthy individuals. Therefore, this study aims to describe gut microbiota development in healthy children from birth till age ten, as well as to investigate potential associations with internalizing and externalizing behavior.

Results: Fecal microbial composition of participants in an ongoing longitudinal study (N=193) was analysed at one, three and four months, and six and ten years of age by 16S ribosomal RNA gene sequencing. Based on these data, three clusters were identified in infancy, two of which were predominated by *Bifidobacterium*. In childhood, four clusters were observed, two of which increased in prevalence with age. One of the childhood clusters, similar to an enterotype, was highly enriched in genus-level taxon *Prevotella* 9. Breastfeeding had marked associations with microbiota composition up till age ten, implying an extended role in shaping gut microbial ecology. Microbial clusters were not associated with behavior. However, *Prevotella* 9 in childhood was positively related to mother-reported externalizing behavior at age ten; this was validated in child reports.

Conclusions: This study validated previous findings on *Bifidobacterium*-enriched and -depleted clusters in infancy. Importantly, it also mapped continued development of gut microbiota in middle childhood. Novel associations between gut microbial composition in the first ten years of life (especially *Prevotella* 9), and externalizing behavior at age ten were found. Replications in other cohorts, as well as follow-up assessments, will help determine the significance of these findings.

Keywords: Infants; children; gut microbiota; development; internalizing behavior; externalizing behavior; *Prevotella* 9

Introduction

The gut microbiota, including a vast number of bacteria (Hillman, Lu, Yao, & Nakatsu, 2017), inhabits the gut of coelomate animals and has co-evolved with the hosts (Nicholson et al., 2012). These resident microorganisms play a crucial role in many aspects of health, including nutrition, immunity and neurophysiology (Cryan et al., 2019; Round & Mazmanian, 2009; Valdes, Walter, Segal, & Spector, 2018). Evidence is accumulating that the gut microbiota also plays a crucial role in aspects of mental health and behavior (Cryan et al., 2019). Therefore, maintaining normal diversity and function of the gut microbiota throughout development is essential for physical and mental health. The current study investigated gut microbial development from infancy to middle childhood, as well as potential relations of the gut microbiota with behavioral measures in healthy children.

In humans, infancy is commonly recognized as a critically important period for microbiota to colonize the gut (Martin et al., 2016). Before weaning, a healthy gut microbiota community is predominated by *Bifidobacterium* (Christian Milani, Sabrina Duranti, Francesca Bottacini et al., 2017). Next to the *Bifidobacterium*-predominated type, researchers have identified several other infant gut microbial types using cluster analyses. The identified clusters are characterized by *Bacteroides*, *Streptococcus*, *Enterobacteriaceae* or *Staphylococcaceae* (Borewicz et al., 2019; Dogra et al., 2015; Matsuki et al., 2016), and are thought to develop as a result of complex extrinsic factors, such as child sex, birth weight, delivery mode, diet, and antibiotics (Cox et al., 2014; Ianiro, Tilg, & Gasbarrini, 2016; Jaggar, Rea, Spichak, Dinan, & Cryan, 2020; Morais et al., 2020; Rutayisire, Huang, Liu, & Tao, 2016; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Stewart et al., 2018; Unger, Stintzi, Shah, Mack, & O'Connor, 2015; J. Wang et al., 2016). Changes in extrinsic factors can also lead individuals to transition between different clusters (Derrien, Alvarez, & de Vos, 2019). While some studies suggested that by age three children have gut microbial profiles that strongly resemble those observed in adults (Koenig et al., 2011; Yatsunenko et al., 2012), other cross-sectional studies concluded that an adult-like gut microbial ecosystem has not yet been completely established at this age (Agans et al., 2011; Hollister et al., 2015; Ringel-Kulka et al., 2013). Compared to healthy adults, the fecal microbiota of healthy toddlers is characterized by a higher relative abundance of *Bifidobacterium* (Ringel-Kulka et al., 2013). Remarkably, *Bifidobacterium* remains more abundant in healthy school-aged children and older-aged adolescents, as compared to healthy adults (Agans et al., 2011; Hollister et al., 2015). All of these cross-sectional studies imply that gut microbiota development may extend longer into childhood than previously thought, potentially due to long-term impacts of early extrinsic factors. However, information on gut microbial development from longitudinal studies is largely lacking in healthy low-risk populations.

Researchers have gradually revealed a bidirectional communication between the gut microbiota and the host brain along the microbiota-gut-brain axis (MGBA), based on accumulating evidence from both animal and human studies mostly focused on clinical mental disorders, such as major depressive disorders and bipolar depression (Aizawa et al., 2016; Crumeyrolle-Arias et al., 2014; Cryan et al., 2019; Hu et al., 2019; Kelly et al., 2016; Lin et al., 2017; R. T. Liu, Walsh, & Sheehan, 2019; Painold et al., 2019; Taylor & Holscher, 2020;

Wallace & Milev, 2017; Zheng et al., 2016). In humans, childhood is regarded as an important phase for behavioral problems to start emerging. Numerous studies showed that elevated childhood behavioral problems, such as internalizing (i.e., behavioral problems influencing children's internal psychological environment, such as anxiety, depression, somatization, and social withdrawal symptoms) and externalizing problems (i.e., behavioral problems manifested in outward behaviour such as antisocial behavior, aggression, hyperactivity, acting out, and hostility) (J. Liu, 2004), are associated with higher chances of developing mental disorders and risky lifestyles in adulthood, which may in turn result in premature mortality (Copeland, Wolke, Angold, & Costello, 2013; Jokela, Ferrie, & Kivimäki, 2009; Mason et al., 2004; von Stumm et al., 2011). However, investigations about the MGBA in childhood are still at an early stage. Most of the existing studies are cross-sectional and mainly focused on children diagnosed with psychological disorders, such as autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD) (Coretti et al., 2018; Jiang et al., 2018; Strati et al., 2017). Three previous longitudinal studies on community samples have reported associations of the gut microbiota with temperament, cognition, and behavioral problems in children until age two years (Aatsinki et al., 2019; Carlson et al., 2018; Loughman et al., 2020). Two papers identified microbial clusters associated with different behavioral patterns (Aatsinki et al., 2019; Carlson et al., 2018), while the third found increased internalizing problems in a *Prevotella*-depleted group (Loughman et al., 2020). However, nearly no studies have longitudinally investigated theses links in populations beyond early childhood.

The present study has two goals. First, we aimed to describe gut microbiota development from birth till age ten in a healthy low-risk community sample. To our knowledge, this has not been done before. We evaluated both short- and long-term associations of the gut microbial composition with extrinsic factors (i.e., birth weight, child sex, delivery mode, breastfeeding, and antibiotics) and determined whether the gut microbiota could be clustered into different successional patterns throughout the first ten years on the basis of variance in microbial composition. Second, we aimed to investigate potential associations of the gut microbiota with internalizing and externalizing behavior in middle childhood in the same cohort. For this second aim, we raised three broad hypotheses based on scarce literature: internalizing and externalizing behavior would (1) differ between microbial clusters; (2) explain general variance in microbial composition; (3) be related to alpha diversity or relative abundances of specific microbial taxa.

Materials and Methods

Participants

Participants were identified from an on-going longitudinal Dutch study named BIBO (Basale Invloeden op de Baby Ontwikkeling) (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011), consisting of healthy low-risk individuals (N=193), with approval from the ethical committee of the Faculty of Social Sciences of the Radboud University (ECG300107, ECG13012012, SW2017-1303-497 and SW2017-1303-498).

Data collection procedures

Parents were instructed to collect fecal samples in sterilized plastic tubes by using the scoop attached to the tube cap, when their children were one, three and four months of age, and six and ten years of age. Infancy samples were collected from diapers, and childhood samples were collected immediately after defecation from potties or toilets without contact with the toilet water. The tubes were then placed in clean plastic bags provided by investigators before temporarily kept in the freezer (-20°C). Samples were transported to the Laboratory of Microbiology at Wageningen University and stored at -80°C before being processed. A total number of 739 fecal samples were collected at these five timepoints. Participants with at least one fecal sample at these assessment moments were included in the present study (N=187).

Behavioral measures were collected with questionnaires at six and ten years of age. Additionally, we recorded the following variables as extrinsic factors that may predict variance in the gut microbiota: child age, child sex, birth weight, delivery mode, frequency of breastfeeding and formula intake in the first 27 weeks of life (mothers were required to weekly record the average number of breastfeeding and formula intake per day), the age of first solid food introduction, and use of antibiotics in the first ten years of life (Beijers, Riksen-Walraven, & de Weerth, 2013; Zijlmans, Beijers, Riksen-Walraven, & de Weerth, 2017). Finally, we also measured dietary intake at age ten by a food frequency questionnaire.

Measures

Gut microbiota composition

In brief, DNA extraction was performed using the Maxwell 16 Total RNA system (Promega, Wisconsin, USA) with 0.01 to 0.13g of fecal sample and Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN), as reported previously (F. Gu et al., 2018). Amplification was performed on the V4 region of 16S ribosomal RNA (rRNA) gene in duplicate, generating amplicons with a length of around 290bp (F. Gu et al., 2018). Each PCR reaction comprised of 10µl of 5xPhusion Green HF Buffer (Thermo Scientific, US), 1µl of 10µM barcoded primers 515F-n(5'-GTGYCAGCMGCCGCGGTAA-3') and 806R-n(5'-GGACTACNVGGGTWTCTAAT-3') (Apprill, McNally, Parsons, & Weber, 2015; Parada, Needham, & Fuhrman, 2016), 1µl of 10mM dNTPs mix (Promega Corporation, US), 0.5µl of 2U/µl Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, US), 36.5µl of Nuclease-free water and 1µl of 20ng/µl DNA template. PCR was carried out as previously described (F. Gu et al., 2018) with modification: initial denaturation (98°C, 30s), 25 cycles of denaturation (98°C, 10s), annealing (50°C, 10s), extension (72°C, 10s) and elongation (72°C, 7min). The presence and length of PCR products was then verified by gel electrophoresis. PCR products were purified by the HighPrep® PCR kit (MagBio Genomics, Alphen aan den Rijn, Netherlands), according to the instructions of the kit. DNA concentration of purified samples was measured using a fluorometer (DS-11; DeNovix) with Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, Netherlands). 200ng of barcoded samples was pooled in libraries comprising 69 uniquely tagged samples, two of which were artificial control communities representative of human gut microbiota (Ramiro-Garcia et al., 2018). The mixture was purified again by HighPrep® PCR kit to a final volume of 40µl.

16S rRNA gene sequencing was completed on the Illumina sequencing platform at Eurofins Genomics, Germany. *NG-Tax* was used for processing of 16S rRNA gene sequence data (Poncheewin et al., 2020; Ramiro-Garcia et al., 2018). Only reads with matching barcodes were kept. Subsequently, amplicon sequence variants (ASVs) were identified on a per sample basis. Taxonomic assignment of ASVs was performed referring to SILVA_132_SSU 16S rRNA gene reference database (Quast et al., 2012). A total of 113,413,327 reads were obtained from the sequencing.

Behavioral measures

CBCL

The Child Behavior Checklist 4-18 (CBCL; for children aged from four to 18) contains 118 specific items of problem behavior, scored on a 3-point scale (Achenbach, 1994). The CBCL includes internalizing and externalizing subscales. Higher scores indicate more behavioral problems. Mothers were required to complete the CBCL when their children were six years old.

SDQ

The Strengths and Difficulties Questionnaire (SDQ) is a 25-item scale, evaluating problem behavior in children from ages four to 16, and scored on a 3-point scale (Goodman, 1997). The SDQ includes internalizing and externalizing subscales. Higher scores represent more behavioral problems. Although the SDQ is shorter than the CBCL, it has verified equivalent ability to assess problem behaviors (Goodman & Scott, 1999). Due to practical reasons, children were asked to complete the SDQ rather than the CBCL when they were ten years old. Mothers also completed the SDQ when their children were ten. Considering known discrepancies between mothers and children in assessing problem behaviors at this age (Van Roy, Groholt, Heyerdahl, & Clench-Aas, 2010), we included both maternal and child reports in the current study.

Questionnaire reliability

To check the internal consistency of the questionnaires, we calculated ω_{total} estimates by using the R package *psych* (Revelle, 2021; Revelle & Condon, 2019). Given ω_{total} estimates were incalculable for the CBCL, we computed Cronbach's α values for this questionnaire instead. The resulting internal consistency estimates were shown as below: the maternal CBCL, $\alpha_{internalizing} = 0.82$, $\alpha_{externalizing} = 0.84$; the maternal SDQ, $\omega_{total\text{-}internalizing} = 0.72$, $\omega_{total\text{-}externalizing} = 0.80$; the child SDQ, $\omega_{total\text{-}internalizing} = 0.63$, $\omega_{total\text{-}externalizing} = 0.59$. Hence, most estimates indicated acceptable or good internal consistency of the subscales. The estimates of the child SDQ were considered questionable, but in line with earlier Dutch studies, and thus used in the analyses (Maurice-Stam et al., 2018).

Extrinsic factors

Extrinsic factors included: (1) Child age when stool samples were collected; (2) Delivery mode (i.e., assisted vaginal delivery, non-assisted vaginal delivery and C-section); (3) Birth weight; (4) Breastfeeding (for samples collected at age one, three and four months, breastfeeding = average number of daily breastfeedings with respect to total number of daily milk feedings (in percentage) until stool collection day; for samples collected at age six and ten years,

breastfeeding = average number of daily breastfeedings with respect to total number of daily milk feedings (in percentage) in the first 27 weeks.); (5) Child age when solid food was first introduced; (6) Child sex (female or male); (7) Total number of antibiotic treatments from birth to stool collection day (infants and children); (8) Whether a child at age six or ten took antibiotics in the past one year (yes or no).

Statistical analyses

All analyses were performed in R (version 3.6.1) (R Core Team, 2020).

First aim: Gut microbiota development in the first ten years of life

Gut microbial clusters and transition patterns

To investigate gut microbiota development, we identified gut microbial clusters based on their compositional features at the genus level by Dirichlet multinomial mixtures (DMM) models, known for their superior advantage of handling sparse data (Holmes, Harris, & Quince, 2012). Considering the reproducibility and stability of the optimal clusters, we split the samples into two parts, infancy (i.e., one, three and four months) and childhood (six and ten years), and performed separate DMM models afterwards. The optimal number of clusters was determined by lowest Laplace approximation scores. As the combination of clusters varied between runnings, we repeated DMM models multiple times and then selected the combinations that appeared the most frequently (Table S1 and Table S2).

Characteristics of gut microbial clusters

The relative abundances of the top 15 predominant genera in infancy and childhood were displayed in heatmaps by the *ComplexHeatmap* package (Z. Gu, Eils, & Schlesner, 2016). Phylogenetic alpha diversity was computed by using the *picante* package (W. Kembel, 2020) and compared between microbial clusters by Wilcoxon rank sum tests. Obtained *p* values from the comparisons were then adjusted by FDR. Beta diversity was compared between microbial clusters by using unweighted or weighted Unifrac distance of genera relative abundances via the *vegan* package (Oksanen, 2020).

Additionally, the functional potential of the microbial community was predicted by Picrust2 (phylogenetic investigation of communities by reconstruction of unobserved states) approach (Douglas et al., 2020; Langille et al., 2013). Predicted gene family counts (i.e., Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs and MetaCyc pathways) for each sample were transferred into relative abundance data. Kruskal-Wallis tests for multiple-group comparisons were first performed on relative abundances of predicted functions between microbial clusters in infancy and childhood, respectively. The predicted function, with an FDR-corrected *p* value lower than 0.05 and the average relative abundance higher than 0.5%, was further compared between every two microbial clusters by Wilcoxon rank sum tests.

Effects of extrinsic factors

First, redundancy analysis (RDA) was used to measure simple effects of extrinsic factors on the gut microbiota for each of five ages (i.e., one, three and four months, and six and ten years), and then infancy (i.e., one, three and four months) and childhood (i.e., six and ten years). Child age, delivery mode, birth weight, breastfeeding and child sex were considered

in infancy. Because only two infants took antibiotics in the first four months of life, we did not consider this factor in infancy RDA models. Similarly, as only one child started consuming solids before the stool collection at four months, this factor was not considered in infancy RDA models. As for samples in childhood, all extrinsic factors mentioned before were included. Quantitative extrinsic factors were converted to z-scores before use.

Second, RDA was performed to measure conditional effects for extrinsic factors with significant simple effects. To avoid potential strong multicollinearity in RDA when assessing conditional effects, we required variance inflation factors (VIFs) of all extrinsic factors to be lower than three (Zuur, Ieno, & Elphick, 2010).

Third, RDA tri-plots were drawn by the *ggplot* package (Wickham, 2016). All RDA models were built based on Bray-Curtis dissimilarity matrices calculated from log-transformed relative abundances at the genus level, via the *vegan* package (Oksanen, 2020). Permutation tests (N = 1000) were used to determine the significance of variance explained by extrinsic factors.

Second aim: Associations of the gut microbiota with internalizing and externalizing behavior in middle childhood

Behavioral differences between microbial clusters

To compare behavioral measures (i.e., internalizing and externalizing behavior) between microbial clusters, we conducted Wilcoxon rank sum tests with FDR adjustment.

RDA models

RDA models were established to determine how much variance in microbial composition could be explained by behavioral measures (i.e., internalizing and externalizing behavior) with and without accounting for extrinsic factors with significant conditional effects. Internalizing and externalizing behavior scores were standardized to z-scores before use. Then, RDA tri-plots were drawn by the *ggplot* package (Wickham, 2016). The same VIF requirements, matrices type and permutation tests as for extrinsic factors were also adopted here.

PRC models

Principle Response Curves (PRC) analysis is a method that enables contrasting time series of experimental groups with a time series of a reference group (van den Brink, den Besten, bij de Vaate, & ter Braak, 2009). Therefore, it was used to select the genera with relatively large differences in relative abundance across ages between behavior groups. Two behavior groups were set for each of the behavior types (internalizing and externalizing behavior). The experimental group (H) included the individuals with behavior scores in the top quartile, while the reference group (L+M) consisted of all remaining individuals. Relative abundance data was pre-processed with log-transformation. PRC models were generated using the *vegan* package (Oksanen, 2020). PRC diagrams were visualized by the *ggplot* package(Wickham, 2016). The first principal component of the variance explained by behavior groups in time, called canonical coefficient, was displayed on the y-axis. The age points were shown on the x-axis. Another vertical axis, named taxon weight, was drawn to elucidate the affinity of the different genera with the response. Wilcoxon rank sum tests with

FDR adjustment were further applied for comparing the differences of selected genera from PRC models between behavior groups at individual time points.

MLM models

Multilevel modelling (MLM) models were conducted to measure the associations of internalizing and externalizing behavior with diversity and selected genera when accounting for extrinsic factors. Selected genera included taxa with absolute values of taxon weights higher than 0.6 and prevalence above 0.2. For MLM models including samples in infancy, extrinsic factors consisted of age, breastfeeding, delivery mode, birth weight and gender. For MLM models in childhood, these factors, as well as antibiotics and age when solid food was introduced, were included. Quantitative extrinsic factors, behavior scores and diversity were converted to z-scores before use. Log transformation was applied to relative abundance data at the genus level. To avoid strong multicollinearity, generalized variance inflation factors (GVIFs) were required to be lower than three. MLM models were built by the *lmerTest* package (Kuznetsova, Brockhoff, Christensen, & Jensen, n.d.).

Significance

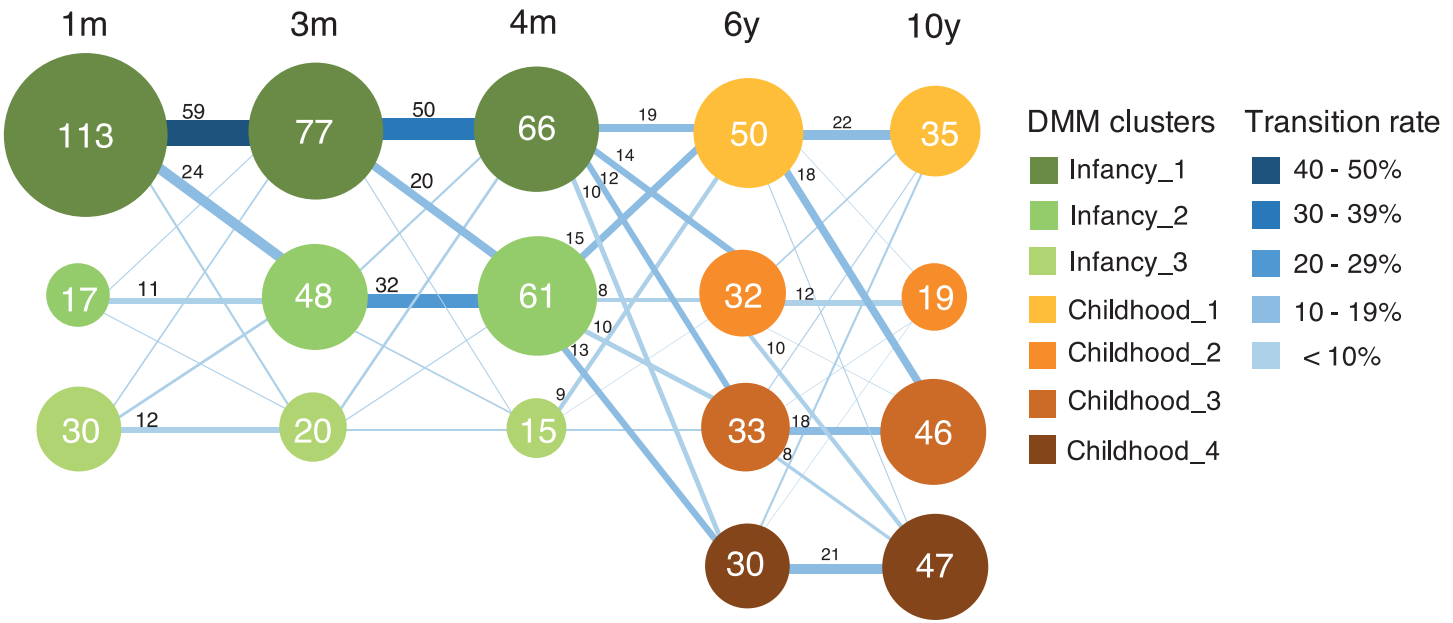
Statistically significant level was required with *p* value lower than 0.05.

Results

First aim: Gut microbiota development in the first ten years of life

Gut microbial clusters and transition patterns

To track gut microbiota development throughout infancy and childhood in the first ten years of life, we stratified the participants into microbial clusters based on their compositional features at the genus level by Dirichlet multinomial mixtures (DMM) models. Based on microbial community composition, three microbial clusters were obtained in infancy, and four clusters were found in childhood (Figure 1). While some individuals maintained the same microbial composition over infancy or childhood, others transitioned to a different microbial cluster when becoming older. At one month of age, 70.6% (113/160) of the infants belonged to Infancy_1, while fecal microbiota of 10.6% (17/160) and 18.8% (30/160) of the infants was classified as Infancy_2 and Infancy_3, respectively. Notably, the prevalence of Infancy_2 continuously increased with age throughout infancy, while the proportions of the other two clusters decreased. From infancy to childhood, no obvious transition pattern was observed. At age six, Childhood_1 included 34.5% (50/145) of the children, while the other three clusters evenly shared the rest. From age six to ten, for individuals belonging to Childhood_1, 44.0% (22/50) remained in the same cluster, and another 36.0% (18/50) converted to Childhood_3. Similar transition patterns were also discerned in Childhood_2, of which 37.5% (12/32) of children remained in the same cluster, and another 31.3% (10/32) transitioned to Childhood_4. Meanwhile, 61.9% (39/63) of the children, belonging to either Childhood_3 or Childhood_4, stayed in the same clusters at age ten. As a consequence, 63.3% (93/147) of children ended up in Childhood_3 and Childhood_4 when reaching age ten.


1m
3m
4m
6y
10y
113
77
66
50
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24
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32
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9
12
18
8
21
DMM clusters
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4
Transition rate
40 - 50%
30 - 39%
20 - 29%
10 - 19%
< 10%

Figure 1. Gut microbial clusters and patterns of transitions between microbial clusters in the first ten years of life. Nodes represent clusters, with colors identifying a compositional cluster. Clusters were identified based on their compositional features at the genus level by Dirichlet multinomial mixtures (DMM) models. The size of the node indicates the number of individuals belonging to this cluster, which is displayed in the node. Lines are sized and colored based on the transition rate, with adjacent numbers representing the number of individuals transitioning from one cluster to another with increasing age. The numbers with transition rates below 6.0% are not shown.

Characteristics of gut microbial clusters

Both Infancy_1 and Infancy_2 clusters were predominated by *Bifidobacterium*, and significantly differed in relative abundances of *Streptococcus*, an unidentified genus within the *Enterobacteriaceae* and *Enterococcus* (Figure 2a). Infancy_1 showed higher relative abundances of *Streptococcus* and an unidentified genus within the *Enterobacteriaceae*, and lower relative abundance of *Enterococcus*, in relation to Infancy_2. Compared to Infancy_1 and Infancy_2, Infancy_3 was depleted in *Bifidobacterium* but enriched in *Streptococcus*, *Enterococcus* and an unidentified genus from the *Enterobacteriaceae*. In childhood, *Bifidobacterium* was among the most predominant genera, albeit at varying relative abundances (Figure 2b). In Childhood_1 *Bifidobacterium* was most predominant as compared to other genera, whereas Childhood_2 was predominated by *Prevotella* 9 at an average relative abundance of 24.5 ± 14.4%, which was much higher than in other clusters (Chilhood_1, 4.1 ± 11.4%; Childhood_3, 0.1 ± 1.0%; Childhood_4, 3.3 ± 4.4%).

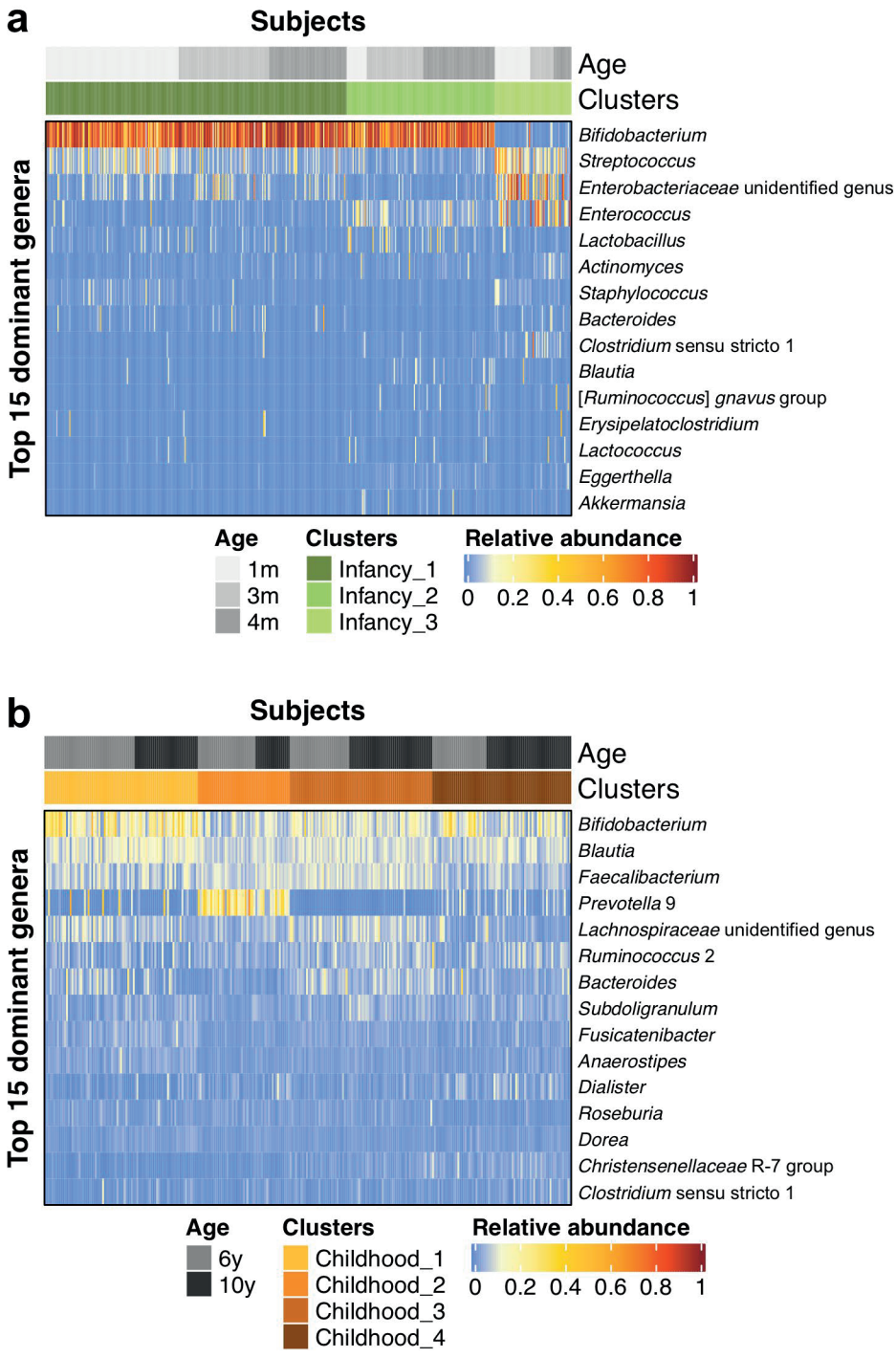

Figure 2. Heatmaps showing the relative abundances of the top 15 predominant genera in the microbial clusters in infancy (a) and childhood (b).

To further describe the features of microbial clusters, we compared the phylogenetic alpha diversity and beta diversity between them (Figure S1 and Figure S2). Significant differences in alpha diversity indices between microbial clusters reflected the results of DMM clustering in the current study.

To describe potential functional differences between microbial clusters, we exploratorily applied the Picrust2 (phylogenetic investigation of communities by reconstruction of unobserved states) method (Douglas et al., 2020; Langille et al., 2013), based on 16S rRNA gene sequence data. In total, 2651 KEGG orthologs and 288 MetaCyc pathways were obtained over the study period. In infancy, 14 KEGG orthologs with average relative abundances higher than 0.5% were significantly different between microbial clusters after FDR adjustment (Table S3), while 13 KEGG orthologs differed significantly in childhood (Table S4). We found the function beta-galactosidase was predicted to be decreased in microbial cluster Infancy_3 (0.19 ± 0.16 %) compared with Infancy_1 and Infancy_2 (0.68 ± 0.17 and 0.67 ± 0.19 %). In later life, beta-glucosidase was observed significantly increased in Childhood_2 (0.84 ± 0.17 %) as compared to the other three childhood microbial clusters (0.67 ± 0.16, 0.62 ± 0.07, and 0.6 ± 0.11 %). Regarding MetaCyc pathways with average relative abundances higher than 0.5%, 72 of them were significantly different after correction in infancy, and 88 differed significantly in childhood. These MetaCyc pathways mainly covered degradation and biosynthesis of carbohydrates and amino acids. In the first several months, pathways of *Bifidobacterium* shunt, mixed acid fermentation, L-arginine biosynthesis Ⅰ and Ⅱ, and superpathway of aromatic amino acid biosynthesis were significantly reduced in Infancy_3 as compared to Infancy_1 and Infancy_2. Pathways of L-arginine biosynthesis Ⅰ and Ⅱ were observed significantly depleted in Childhood_2 as compared to the other three microbial clusters in childhood.

In addition to microbial compositional features, we also exploratorily investigated whether microbial clusters differed on population characteristics, namely delivery mode, child sex, breastfeeding, medications, diseases and etc., in infancy and childhood (Table S5 and Table S6). Delivery mode was significantly different between infant microbial clusters, of which Infancy_3 showed highest rates in C-section and assisted vaginal delivery. Food frequency was also compared between microbial clusters in childhood, and no significant differences were observed (Table S7).

Associations of gut microbial composition with extrinsic factors

To determine to what extent extrinsic factors (i.e., birth weight, child sex, delivery mode, breastfeeding, and antibiotics) can explain the observed variation in microbiota composition, their simple effects (i.e., the impact of one factor on gut microbiota without taking other factors into account) were measured separately per time point, as well as for infancy and childhood samples, respectively (Table S8 and Table S9). In infancy, breastfeeding significantly explained 1.0%, 1.4% and 1.2% of adjusted variances without the biases in microbial composition, in separate analyses at age one, three and four months. None of the other factors tested, i.e., delivery mode, birth weight and child sex, significantly contributed to explaining the observed variation in microbial composition, in separate analyses at infancy time points. In childhood, no significant simple effects were observed at single time points.

In analyses pooling all infancy samples together, child age was found having the most predominant significant effect (1.0%), followed by breastfeeding and delivery mode (0.7% and 0.2%). With respect to simple effects of extrinsic factors in childhood, breastfeeding significantly explained around 0.3% of adjusted variance in microbial composition in the pooled data of ages six and ten. Similar to infancy, in the period from age six to ten, child age significantly explained the most observed variance in microbial composition (0.9%) as compared to other extrinsic factors of which only breastfeeding explained significant variance (0.3%).

Next to it, we measured conditional effects (i.e., the impact of individual factors when partitioning out effects from other factors) of the significant extrinsic factors obtained from pooled data (Table 1). These extrinsic factors included: (1) Child age, delivery mode, and breastfeeding for infancy; (2) Child age and breastfeeding for childhood. After partitioning out total explained variance, selected infancy and childhood extrinsic factors were still able to significantly explain partial variance.

Table 1. Conditional effects of extrinsic factors with significant simple effects on gut microbiota in infancy and childhood.

	R2%	Adjusted R2%	VIF	Number of individuals	Number of genera
1-4m					
Age	1.051	0.817	1.017		
Delivery mode	0.639	0.16	1.724 (CS); 1.723 (NAVD)	411	155
Breastfeeding	0.791	0.556	1.017		
6-10y					
Age	1.554	0.869	1.001	144	181
Breastfeeding	1.017	0.329	1.001		

Significance was determined based on 1000 permutations. Asterisks indicate p value < 0.05. VIF: variance inflation factor. CS: C-section. NAVD: non-assisted vaginal delivery.

To gain more insights into the associations between the gut microbiota and extrinsic factors with significant conditional effects, we performed analyses on the pooled infancy data and the pooled childhood data (Figure 3). During infancy, breastfeeding was positively associated with increased relative abundances of *Bifidobacterium* and an unidentified genus within the *Enterobacteriaceae*. Infants with C-section showed higher levels of *Streptococcus* and *Enterococcus*, and lower levels of *Bifidobacterium*. With increasing age from one to four months, *Bifidobacterium*, *Actinomyces* and *Eggerthella* also increased in relative abundances. Over childhood, age was positively related to higher relative abundances of unidentified genera from the *Ruminococcaceae* and *Peptostreptococcaceae*. In the same age period,

higher breastfeeding was associated with higher relative abundances of *Prevotella* 9 and *Dialister*.

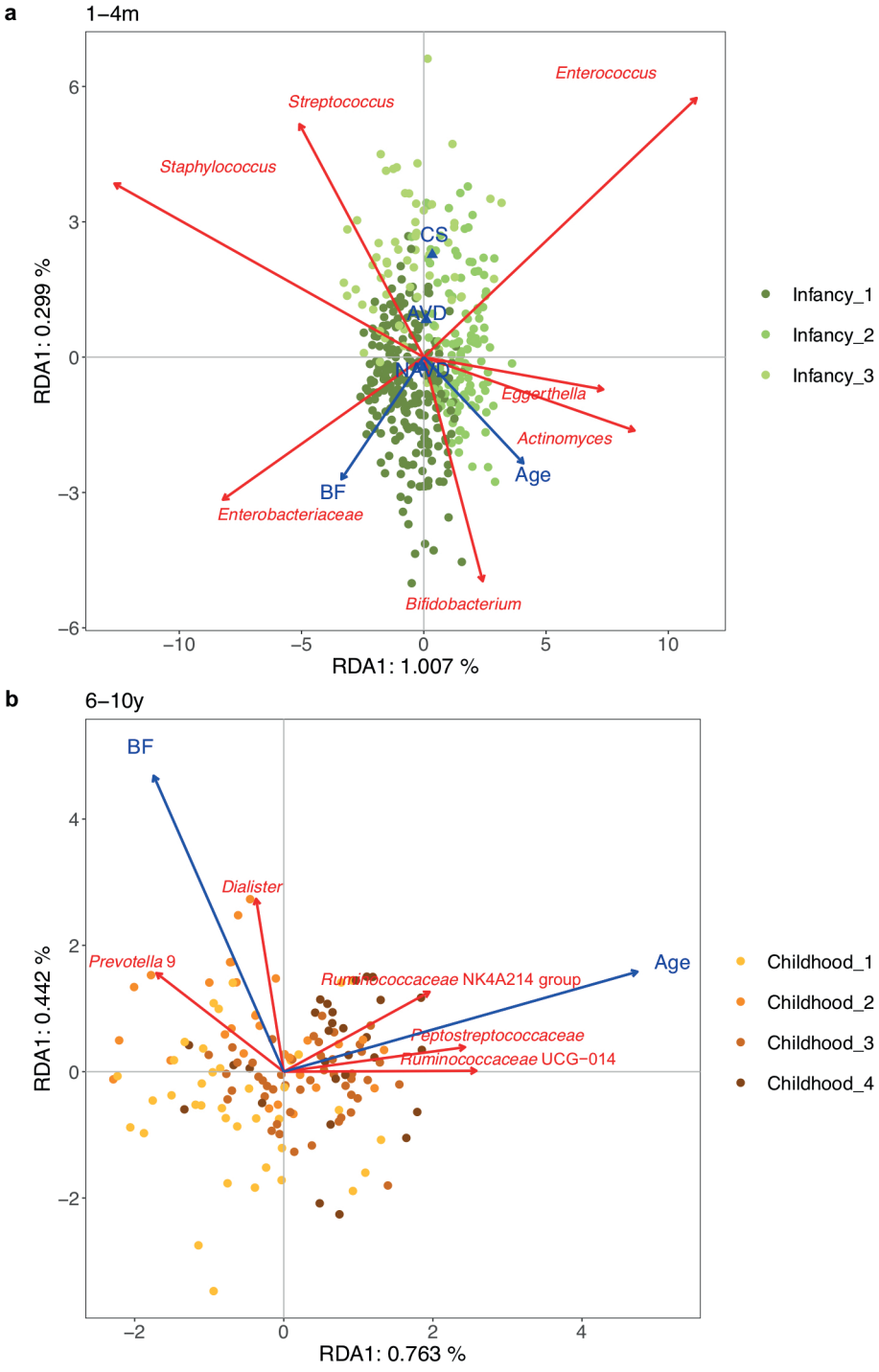

Figure 3. RDA plots of extrinsic factors with significant conditional effects on gut microbial composition. (a) RDA plot based on microbiota profiles at the age of one, three and four months. (b) RDA plot based on microbiota profiles at the age of six and ten years. RDA plots are displayed based on Bray-Curtis distance matrices computed from log-transformed data at the genus level. Unidentified genera are shown at the family level. To clarify, we displayed adjusted variances along axes, which were corrected to be without biases. Generally, the value of adjusted variances is lower than that of original variances. CS: C-section. AVD: assisted vaginal delivery. NAVD: non-assisted vaginal delivery. BP: breastfeeding proportion.

Second aim: Associations of the gut microbiota with internalizing and externalizing behavioral measures in middle childhood

Microbial clusters and behavior

We did not find any significant associations between microbial clusters and child internalizing and externalizing behavior (Figure S3 and Figure S4).

RDA models

Before exploring associations between the gut microbiota and behavioral measures, we first assessed which behavioral measures were capable of significantly explaining variance in microbial composition with and without accounting for the extrinsic factors studied in the first aim, by using Redundancy analysis (RDA) models. Without accounting for these factors, internalizing behavior, evaluated by the Strengths and Difficulties Questionnaire (SDQ) maternal reports at ten years of age, was able to explain the variance in microbial composition at one month of age (p = 0.050; Table S10).

As for samples in childhood (Table S11), maternal-reported externalizing behavior, measured by the SDQ at age ten, significantly explained variance in gut microbial composition at age six. Remarkably, we found both internalizing and externalizing behavior, assessed by the maternal SDQ at age ten, were able to significantly explain variation in the gut microbiota in the period from age six to ten. When taking also significant extrinsic factors into account, i.e., child age and breastfeeding (Table 2), we found that the maternal SDQ reports of externalizing behavior at age ten still significantly explained variance in microbial composition during childhood. However, internalizing behavior was no longer significant in this model.

Table 2. Partial variance in microbial composition in childhood explained by behavioral problems at age ten as reported by the mother (SDQ).

	Behavior	R2%	Adjusted R2%	*p* value	VIF	Number of genera
6-10y						
SDQ_M_10y	INT	0.851	0.127	0.148	1.062	181
	EXT	1.098	0.379	0.005*	1.058	

Significance was determined based on 1000 permutations. Asterisk indicates p value < 0.05. VIF: variance inflation factor. CS: C-section. NAVD: non-assisted vaginal delivery.

To specifically explain the associations of pooled gut microbiota of ages six and ten with internalizing and externalizing behavior assessed by the maternal SDQ at age ten, partial RDA was performed by accounting for age and breastfeeding (Figure 4). Externalizing behavior showed positive associations with relative abundances of *Prevotella* 9 and *Phascolarctobacterium*. In addition, more internalizing behavior was related to reduced relative abundance of *Akkermansia*, and more externalizing behavior was associated with decreased relative abundance of *Alistipes*. Finally, a higher relative abundance of *Terrisporobacter* was found in individuals with higher internalizing behavior scores.

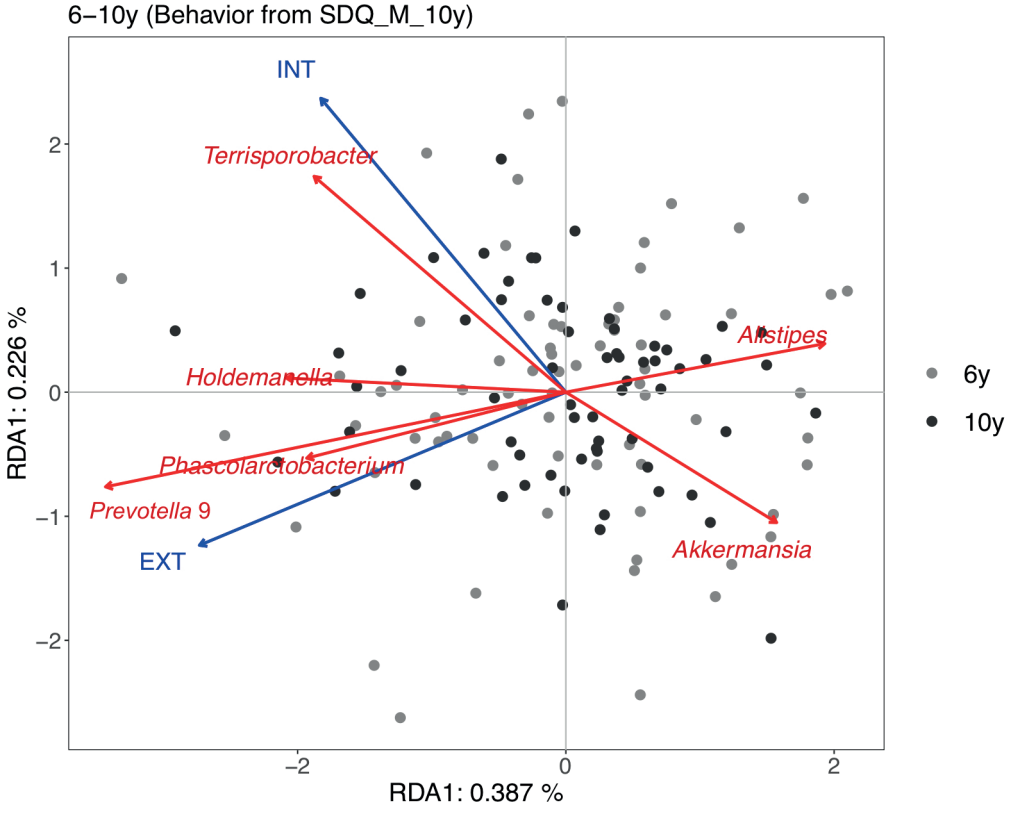

Figure 4. Partial RDA plot indicating associations of genera in childhood with internalizing and externalizing behavior at age ten as reported by the mother (SDQ). RDA plots are displayed based on Bray-Curtis distance matrices calculated from log-transformed data at the genus level. Child age and breastfeeding were accounted for. To clarify, we displayed adjusted variances along axes, which were corrected to be without biases. Generally, the value of the adjusted variance was lower than that of the original variance. INT: internalizing behavior. EXT: externalizing behavior.

PRC models

In order to assess emerging associations of gut microbiota composition as measured during early infancy and childhood with internalizing and externalizing behavior at age ten, we

performed Principle Response Curves (PRC) analyses (Figure 5). This method can be used to assess temporal trajectories of dissimilarity between behavior groups with different scores, and to select genera with relatively large changes in relative abundances across the first ten years of life. With respect to internalizing behavior, measured by the maternal SDQ at age ten, no obvious differences were observed between high (H) and low / medium (L+M) score groups in the first four months of life, whereas the dissimilarity in microbial composition between groups started changing somewhere between four months and six years.

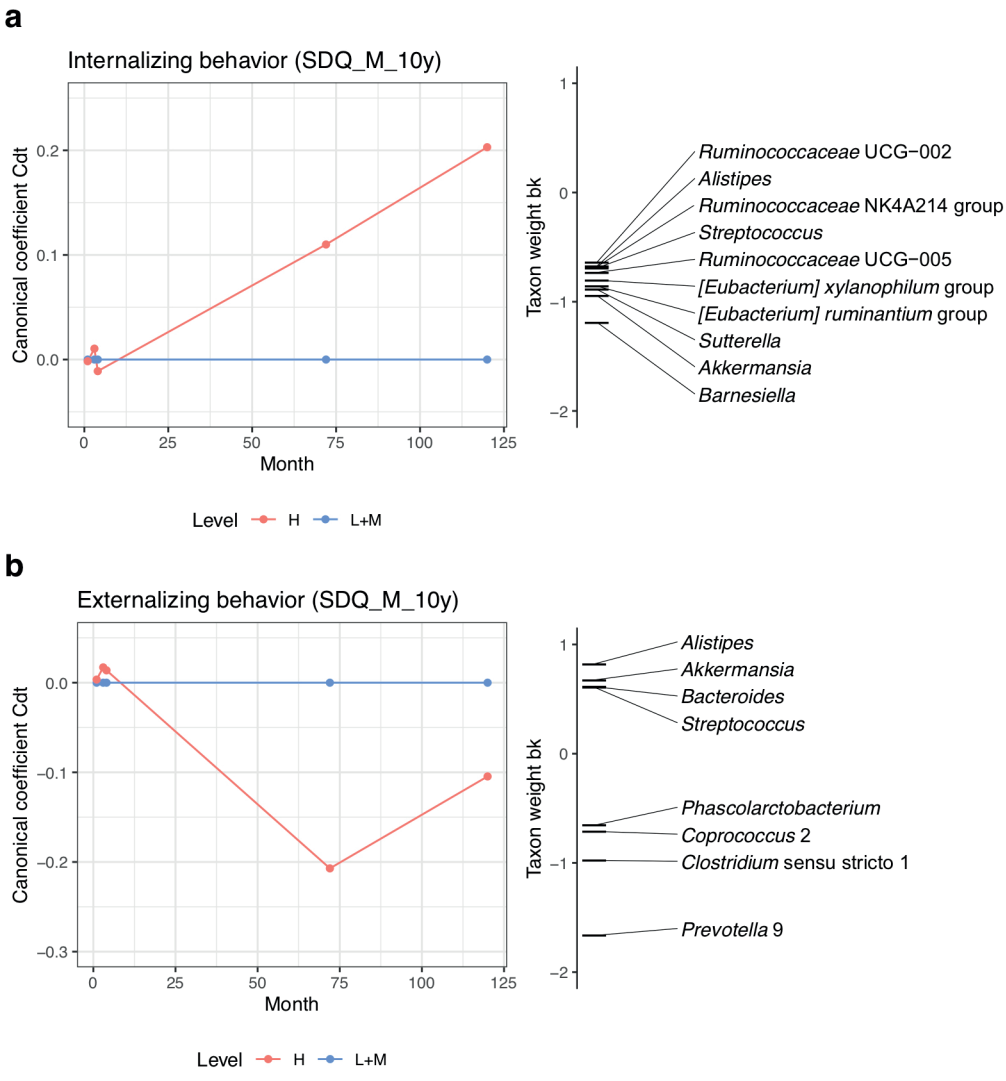

Figure 5. Principle Response Curve analysis of internalizing (a) and externalizing behavior (b) at age ten as reported by the mother (SDQ). Behavior groups are set based on quartiles. H level includes individuals with scores in the top quartile, and L+M level includes the bottom three quartiles. L+M level is used as reference (Low+Medium; baseline). Canonical coefficients indicate the differences between H and L+M. The wider the distance between H and L+M, the more dissimilar they are to each

other. The taxon weight reflects for which taxa the compositional variation is best represented by the PRC model. The direction of abundance change is determined jointly by the signs of canonical coefficient and taxon weight. Same signs indicate increased relative abundance, while reverse signs represent reduced relative abundance. Genera with absolute values of taxon weights lower than 0.60 are not displayed. Genera data was pre-processed with log-transformation.

Similarly, with respect to externalizing behavior, measured by the same questionnaire at the same age, differences between behavior groups started emerging between four months and six years. Among the time points included in this study, the difference between groups of externalizing behavior was highest at age six, and then decreased again at age ten. Children belonging to the top quartile (H), tended to show higher levels of *Prevotella* 9 and *Clostridium* sensu stricto *1*, and reduced relative abundances of the genera *Alistipes*, *Akkermansia*, *Bacteroides* and *Streptococcus* (Figure 5). As the coefficients for group H are negative compared to the baseline, negative taxon weights indicate positive correlations.

Furthermore, we compared the differences in relative abundances of the genera selected from PRCs between behavior groups at each age. With respect to internalizing behavior, the genera *Akkermansia*, *Alistipes*, *Sutterella*, *Barnesiella*, and two genus-level groups of *Eubacterium* were observed reduced in individuals belonging to the H group at age ten, although not significantly after FDR adjustment. As for externalizing behavior, *Alistipes* was depleted, and *Phascolarctobacterium*, *Coprococcus* 2, *Clostridium* sensu stricto 1 and *Prevotella* 9 were increased in relative abundances in the H group at age six. After FDR correction, *Clostridium* sensu stricto 1 and *Prevotella* 9 were observed with *p* values lower than 0.10. At age ten, the relative abundance of *Clostridium* sensu stricto 1 remained higher in H group individuals, albeit insignificant after FDR correction.

MLM models

Multilevel models (MLM) were used due to their ability in processing time-series data. These models included the following extrinsic factors: child age, delivery mode, breastfeeding, birth weight, and child sex in infancy, and the same factors, as well as age of solid food introduction and antibiotic treatments, in childhood. For the maternal SDQ at age ten, we found that internalizing behavior was moderately positively related to phylogenetic alpha diversity during infancy (Table 3), while externalizing behavior was not related to alpha diversity or relative abundances of specific genera in infancy. In childhood, *Alistipes* was found to be significantly negatively associated with externalizing behavior assessed by the maternal SDQ at age ten. In addition, higher relative abundances of *Prevotella* 9 and *Phascolarctobacterium* were significantly associated with increased externalizing behavior. Interestingly, similar associations of *Prevotella* 9 and *Phascolarctobacterium* with externalizing behavior were further validated in the SDQ child reports at age ten (Table S12). MLM models were also conducted for behavior assessed by the maternal CBCL at age six, however, no consistent associations were found (Table S13).

Table 3. MLM models for internalizing and externalizing behavior at age ten as reported by the mother (SDQ).

	SDQ_M_10y_Int			SDQ_M_10y_Ext		
Response variable	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
1-4m						
Phylogenetic diversity	0.127	[-0.014, 0.269]	0.088	-0.052	[-0.189, 0.086]	0.473
Streptococcus	0.08	[-0.075, 0.234]	0.327	-0.081	[-0.232, 0.071]	0.31
Clostridium sensu stricto 1	-0.047	[-0.232, 0.138]	0.628	0	[-0.179, 0.180]	0.999
6-10y						
Phylogenetic diversity	-0.054	[-0.248, 0.139]	0.61	0.084	[-0.110, 0.278]	0.426
Streptococcus	0.166	[-0.078, 0.407]	0.21	-0.192	[-0.436, 0.047]	0.147
Clostridium sensu stricto 1	0.18	[-0.032, 0.393]	0.122	0.186	[-0.025, 0.397]	0.107
Bacteroides	0.089	[-0.077, 0.253]	0.323	-0.172	[-0.338, -0.008]	0.058
Barnesiella	-0.181	[-0.440, 0.079]	0.2	-0.143	[-0.402, 0.114]	0.309
Prevotella 9	0.222	[-0.194, 0.637]	0.326	0.614	[0.192, 1.035]	0.009*
Alistipes	-0.088	[-0.319, 0.148]	0.489	-0.333	[-0.566, -0.101]	0.010*
Coprococcus 2	-0.024	[-0.289, 0.231]	0.863	0.172	[-0.083, 0.427]	0.219
Ruminococcaceae NK4A214 group	0.034	[-0.184, 0.250]	0.774	-0.145	[-0.355, 0.072]	0.212
Phascolarctobacterium	0.065	[-0.224, 0.355]	0.677	0.339	[0.046, 0.632]	0.036*
Sutterella	-0.117	[-0.365, 0.124]	0.378	0.101	[-0.145, 0.341]	0.448
Akkermansia	-0.254	[-0.537, 0.033]	0.103	-0.158	[-0.439, 0.123]	0.304
[Eubacterium] ruminantium group	-0.097	[-0.321, 0.128]	0.428	0.046	[-0.174, 0.270]	0.703
[Eubacterium] xylanophilum group	-0.21	[-0.415, -0.005]	0.063	0.071	[-0.131, 0.271]	0.52
Ruminococcaceae UCG-002	-0.095	[-0.303, 0.107]	0.396	-0.056	[-0.257, 0.149]	0.615
Ruminococcaceae UCG-005	-0.077	[-0.349, 0.199]	0.606	-0.116	[-0.389, 0.158]	0.435

Numbers of individuals included in MLM models were listed in **Table S14**. Genera with prevalence lower than 0.20 were not included in MLM models. Detailed information of genera prevalence can be reached in **Table S15**. Asterisk indicates *p* value < 0.05.

Discussion

In our study, three distinct microbial clusters were identified in samples taken during the first four months of life. This is in line with previous studies focusing on the first half year of life (Borewicz et al., 2019; Dogra et al., 2015; Matsuki et al., 2016), both for the number of clusters and the microbial cluster composition. Regarding Infancy_1 and Infancy_2, they were predominated by *Bifidobacterium* with numerical predominance values at 77.5 ± 20.9% and 75.2 ± 20.4%, which are similar to the numerical predominance values of *Bifidobacterium*-predominated clusters in the three previous studies (Borewicz et al., 2019; Dogra et al., 2015; Matsuki et al., 2016). Contrary to the *Bifidobacterium*-enriched clusters, Infancy_3 was depleted in *Bifidobacterium* but enriched in *Streptococcus* and an unidentified genus within *Enterobacteriaceae*; these characteristics were also found in previously reported clusters (Borewicz et al., 2019; Dogra et al., 2015; Matsuki et al., 2016). Notably, the identification of these *Bifidobacterium*-depleted clusters varies between studies. For instance, Dogra et al. found two, rather than one, *Bifidobacterium*-depleted clusters, enriched in *Streptococcus* and *Enterobacteriaceae*, respectively (Dogra et al., 2015). This may be due to the differences in clustering methods and study populations. Also, Borewicz et al. described a *Bacteroides*-predominated cluster that was absent in our study (Borewicz et al., 2019). High intra-individual variability of the gut microbiota may explain these different findings, since we used the same clustering method and included participants from the same country as Borewicz et al. Future large-scale studies and meta-analyses may help clarify these clustering issues in infant populations.

Remarkably, the infant cluster transition patterns in our study were highly similar to those previously reported by other studies (Dogra et al., 2015; Hill et al., 2017). The prevalence of *Bifidobacterium*-enriched clusters was increased from one to four months of age, while the ratio of Infancy_3 was reduced. Infancy_3 was identified with the highest proportion of C-section and assisted vaginal deliveries, while no differences in breastfeeding were observed between infant clusters in our study. The shift towards a *Bifidobacterium*-enriched microbial community in infants from the Infancy_3 cluster may imply a quick adaption to environmental changes, such as the initiation of breastfeeding. Given the similarity between Infancy_1 and Infancy_2, generating more specific profiles of *Bifidobacterium* species and strains may help enhance their differentiation. In sum, our study provides further support on the consistency of gut microbial clusters in infancy, regardless of differences between studies in sample size, collection period and clustering method.

In fecal samples taken at six and ten years of age, we distinguished four microbial clusters and delineated how children transitioned between these clusters, with two clusters separately predominated by *Bifidobacterium* and *Prevotella* 9 and the other two enriched in *Bifidobacterium*, *Blautia* and *Faecalibacterium*. Our clusters showed similarities and differences to those of a recent study by Zhong et al. in healthy Dutch school-aged children

(mean age 7.3 years, ranging from six to nine) (Zhong et al., 2019). Our Childhood_1 closely resembled the *Bifidobacterium*-dominated cluster reported by Zhong et al. and showed a similar relative abundance of *Bifidobacterium* at 21.6 ± 12.0%. In adults, the relative abundance of *Bifidobacterium* normally ranges from 2-14% (Odamaki et al., 2016). The other three childhood clusters observed in our study were within this range. Although a *Bifidobacterium*-predominated microbiota is commonly known to be beneficial for infants, this type of cluster may lack maturity in children and adults (Derrien et al., 2019). Moreover, Childhood_1 displayed the lowest diversity among all four clusters (Fig. S1; also reported by Zhong *et al*). This finding further supports the notion of immaturity of Childhood_1, as the lower diversity may be paired to a corresponding lower functional potential that may not fully meet the requirements of complex carbohydrate utilization and butyrate production in later life (Zhong et al., 2019). Childhood_2 was similar to a *Prevotella*-predominated cluster observed by Zhong *et al*., and also to one of the three human adult enterotypes (Arumugam et al., 2011). These microbial community types exhibit approximately 20% of *Prevotella*, a genus positively associated with carbohydrate intake and fiber consumption (De Filippo et al., 2010; Wu et al., 2011). In contrast to the findings of Zhong *et al*., in our study, no *Bacteroides*-predominated cluster was found. Although Childhood_3 showed the highest level of *Bacteroides* across our clusters, the relative abundance of this genus (6.6 ± 4.4%) was lower than in the corresponding community type reported by Zhong et al. (near 20%). Childhood_3 was enriched in a group of near evenly distributed genera, including *Bifidobacterium*, *Blautia*, *Faecalibacterium*, and an unidentified genus within *Lachnospiraceae* (12.3 ± 6.2%, 10.2 ± 3.1%, 10.0 ± 3.5% and 8.49 ± 5.6%). Childhood_4 had similar levels of *Bifidobacterium* and *Blautia* (13.3 ± 10.2% and 8.9 ± 4.2%) as Childhood_3, while comprising lower levels of *Faecalibacterium* and an unidentified genus within *Lachnospiraceae* (5.9 ± 3.2% and 3.7 ± 4.4%) than Childhood_3. Both Childhood_3 and Childhood_4 showed more diverse and more evenly distributed microbiota than *Bifidobacterium*-predominated Childhood_1 and *Prevotella* 9-predominated Childhood_2; these features may allow more complex functions in Childhood_3 and Childhood_4, and hence may mark a mature gut microbiota community for children in middle childhood or at a later age.

The differences between the studies may be attributed to age, as Zhong et al. included consecutive time points from age six to nine, while the present study was specifically focused on ages six and ten. This particular period, spanning four years and reaching into early puberty, may be of relevance for gut microbial development. Indeed, from age six to ten, we observed an overall progressive transition of children from Childhood_1 and Childhood_2 to Childhood_3 and Childhood_4, both displaying higher alpha diversity than the other two clusters, hinting at a trend towards increasing microbial functional capacity from age six to ten. This also indicates that in healthy children gut microbial development appears to continue at least until early puberty. Though diet is regarded as an important factor influencing the gut microbiota, we did not find differences between childhood microbial clusters with respect to the children's dietary intake. Note, however,

that this may be due to the fact that we only measured food frequency at age ten, while the gut microbiota was assessed at ages six and ten.

We further investigated potential functional differences of the gut microbiota between microbial clusters in an exploratory manner by using the Picrust2 approach. In general, we noticed that multiple predicted metabolic functions (i.e., KEGG orthologs and MetaCyc pathways) varied between microbial clusters in infancy and childhood. For example, in infancy, we observed that the level of KEGG ortholog beta-galactosidase, an enzyme catalyzing the hydrolysis of beta-galactosides like lactose, was lower in Infancy_3 in comparison with the other two infant microbial clusters. Beta-galactosidase has been found prevalent in *Bifidobacterium* species (Hsu, Yu, & Chou, 2005). Consistent with this, Infancy_3 showed the lowest level of *Bifidobacterium*; hence, the depletion of *Bifidobacterium* may explain the reduction of beta-galactosidase in Infancy_3. In childhood, we found that the relative abundance of the KEGG ortholog beta-glucosidase, an enzyme hydrolyzing various glycosides like cellulose coming from plant foods, was highest in microbial cluster Childhood_2. Childhood_2 was enriched in a fiber-favoring bacterium *Prevotella* 9. As a consequence, this cluster can be considered to have a higher ability of utilizing cellulose, which is in line with our finding. As for differences in MetaCyc pathways, we observed that the biosynthesis of precursors (i.e., aromatic amino acids) for neurotransmitters (i.e., serotonin, dopamine and norepinephrine), was decreased in Infancy_3. This microbial cluster also showed decreases in mixed acid fermentation and *Bifidobacterium* shunt, which might indicate a reduction in short-chain fatty acids (SCFAs) production. Though the role of SCFAs on the MGBA has not been clearly elucidated, they are speculated to have considerable impacts (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019). In both Infancy_3 and Childhood_2, we noticed decreased levels in predicted functions of L-arginine biosynthesis I and II. L-arginine supplementation has been reported to stimulate glutamate decarboxylation in *Lactococcus lactis*, which in turn increases the production of the neurotransmitter gamma-aminobutyric acid (GABA) (Laroute et al., 2016). However, it is unknown if other microbial taxa have similar interactions of L-arginine with GABA. Finally, note that there are two main limitations of any function prediction tool based on marker genes such as Picrust2 (Douglas et al., 2020). The first is the bias caused by the reference database, and the second is that the resolution cannot distinguish strain-specific functionality. Hence, these findings of predicted functions must be seen as exploratory and interpreted with caution.

Regarding extrinsic factors, breastfeeding was found to explain a moderate amount of variance in infant gut microbial composition, similarly to our previous findings in which breastfeeding explained 2-6% of the variance (Zijlmans, Korpela, Riksen-Walraven, de Vos, & de Weerth, 2015). In line with previous studies (M. A. E. Lawson et al., 2020; Stewart et al., 2018; Yatsunenko et al., 2012), increased breastfeeding was found related to higher levels of *Bifidobacterium* in the first four months of life. Surprisingly, early-life breastfeeding was also associated with the gut microbiota in the period from six to ten years of age. This finding tied well with observations by Zhong *et al.*, who uncovered a persistent effect of breastfeeding duration on the gut microbiota based on community samples at school age

(Zhong et al., 2019). Although it is widely accepted that breastfeeding only prominently affects the gut microbiota in infancy or early childhood (O'Callaghan & van Sinderen, 2016; Stewart et al., 2018), both Zhong's and our findings may indicate an extended influence of breastfeeding on shaping microbial composition and even function. In addition, we found that breastfeeding was positively associated with increased *Prevotella* 9 in childhood. *Prevotella*, as a genus prevalent in populations consuming fiber (De Filippo et al., 2010), has been found tightly linked to glucose metabolism (Kovatcheva-Datchary et al., 2015). However, in this study, it is unknown if the increased level of *Prevotella* 9 is caused by breastfeeding or other relevant dietary factors. In a recent study based on another population, we found that longer exclusive breastfeeding duration was associated with a healthier child diet at age three years (Willemsen, Beijers, Arias Vasquez, & de Weerth, 2021), note though that, as mentioned before, diet at age ten was unrelated to child gut microbiota. Further studies aiming to validate this association and explore causality are hence needed to clarify these issues.

Regarding associations between the gut microbiota and child behavior, we found no associations of the microbial clusters with internalizing and externalizing behavior measured by maternal and child reports at age six and ten. In earlier studies, clustering methods were also adopted with the aim of exploring links of the child gut microbiota with subsequent temperament at six months and cognition at two years (Aatsinki et al., 2019; Carlson et al., 2018). Compared to these studies in which the microbial composition was analyzed at one selected time point, in the present study we used five time points in the first ten years of life to more comprehensively delineate relations between the microbial clusters and problem behavior. Although we did not find that microbial clusters were related to problem behavior in our study, this does not imply that clustering methods were inappropriate to use. Indeed, clustering methods are highly suitable for high-dimensional data. Also, it is worth noting that there can be a moderate relation between the gut microbiota and problem behavior, the substantiation of which might require larger datasets to reflect this relation. Furthermore, variation in microbial composition does not directly provide information about differences in microbial function involved in MGBA. In other words, different microbial communities may hold similar gene potential. Limited by the 16S rRNA sequencing technique, we were only able to explore function with the Picrust2 method in the current data. This method has shortcomings that can be avoided by using metagenomics in combination with metabolomics analyses in future studies.

With respect to specific microbial taxa, based on several complementary statistical methods, including RDA, PRC and MLM models, we found that the relative abundances of *Prevotella* 9 and *Phascolarctobacterium* in samples taken at age six to ten were positively associated with increased mother-reported externalizing behavior at age ten, while a negative association was observed in the level of *Alistipes* with the same externalizing behavior at the same age. A previous longitudinal study in toddlers found that a higher relative abundance of *Prevotella* at one year of age was related to more problem behavior, particularly internalizing behavior (Loughman et al., 2020). The large age gap and different assessment moments may explain the differences between the two studies. Comparing our

findings with studies focusing on children with psychopathology, we find that autistic children (four to 11 years), commonly exhibiting co-occurring externalizing problems (Baker et al., 2018), showed increased abundances of microbial groups including *Prevotella*, *Bacteroides* and *Porphyromonas*, compared to healthy controls (De Angelis et al., 2013). In contrast, another study found that *Prevotella* was reduced in children with autism (three to 16 years) (Kang et al., 2013). Apart from autism, ADHD has also been shown to be associated with externalizing behavior in adolescence (Kuja-Halkola, Lichtenstein, D'Onofrio, & Larsson, 2015). For children with ADHD, two previous studies reported no changes in *Prevotella* abundance (Jiang et al., 2018; L.-J. Wang et al., 2020), whereas Kristensen et al. found decreased levels of *Prevotellaceae* (Prehn-Kristensen et al., 2018). In sum, there is no well-defined link between *Prevotella* and behavioral problems and mental disorders, just as at the physical health level, *Prevotella* has been related to the consumption of beneficial plant-rich diets, but also to harmful chronic inflammation (Ley, 2016). As a large genus, more than 50 characterized species have been identified within *Prevotella* so far, greatly varying in their genetic potential (Ley, 2016; Tett, Pasolli, Masetti, Ercolini, & Segata, 2021). In this case, using metagenomic analyses to characterize the *Prevotella* population at higher taxonomic resolution, i.e., species or strain level, would be helpful to better understand a more specific potential interaction with host behavior. With respect to *Phascolarctobacterium*, a systematic review showed its relative abundance was higher in patients with major depressive disorder (MDD) than controls (Cheung et al., 2019), while Li et al. reported that it was positively related to improved mood in adults with the same dietary structure (Li et al., 2016). Though these studies reflect that *Phascolarctobacterium* is related to internalization-relevant mental problems, it is worth noting that internalizing and externalizing behavior can co-exist in children and may lead to opposite behavioral problems at a later age (Aronen & Soininen, 2000; McConaughy & Skiba, 1993; Zoccolillo, 1992). As for *Alistipes*, earlier studies found its role was divergent in MDD and ASD (Cheung et al., 2019; De Angelis et al., 2013; Strati et al., 2017). As described before, distinct behavioral issues can co-occur and even predict the opposite one in the same child; this does not only work for MDD but also for ASD which is often accompanied by greater aggression (R. A. Lawson et al., 2015). Given the complexity of mental problems, the associations of *Phascolarctobacterium* and *Alistipes* with externalizing behavior need to be interpreted with caution.

Strengths of this study include the prospective longitudinal design with repeated gut microbial sampling in healthy community children. Additionally, behavioral measures were reported by both mothers and children and at two different ages, and a series of sophisticated and complementary statistical analyses were performed. A limitation of the study is the restricted taxonomic resolution of the 16S rRNA gene sequence data used in this study, that does not permit us to distinguish microbial taxa at the species or strain level.

In sum, in this study we identified three microbial clusters in infancy and four in childhood and explored transitional trajectories of individuals through these clusters in the first ten years of life. These clusters exhibited similarities as well as differences to previously identified clusters. Among the different extrinsic factors studied, breastfeeding stood out by

having marked associations with the gut microbiota up till age ten, implying an extended role in shaping gut microbial ecology. With respect to problem behavior, high relative abundances of *Prevotella* 9 and *Phascolarctobacterium* and a low level of *Alistipes* in middle childhood were associated with increased externalizing behavior at age ten. In the future, strain-resolved metagenomic sequencing, as well as specific sets of qPCR assays, can provide a better understanding of the potential role of *Prevotella* 9 in child behavior. Additionally, determining behaviorally relevant fecal metabolites will help bridge the gap between association and causality. Finally, to take a step further in understanding the development of the gut microbiota throughout childhood, as well as its relations with child behavioral phenotypes, healthy longitudinal cohorts with a higher frequency of gut microbial sampling (e.g., yearly samples throughout childhood) are direly needed.

Data availability statement

As the findings in this study are supported by datasets from an ongoing longitudinal cohort, these datasets currently cannot be made publicly available but are available upon request from C.deW. (Carolina.deWeerth@radboudumc.nl)

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Supplemental information

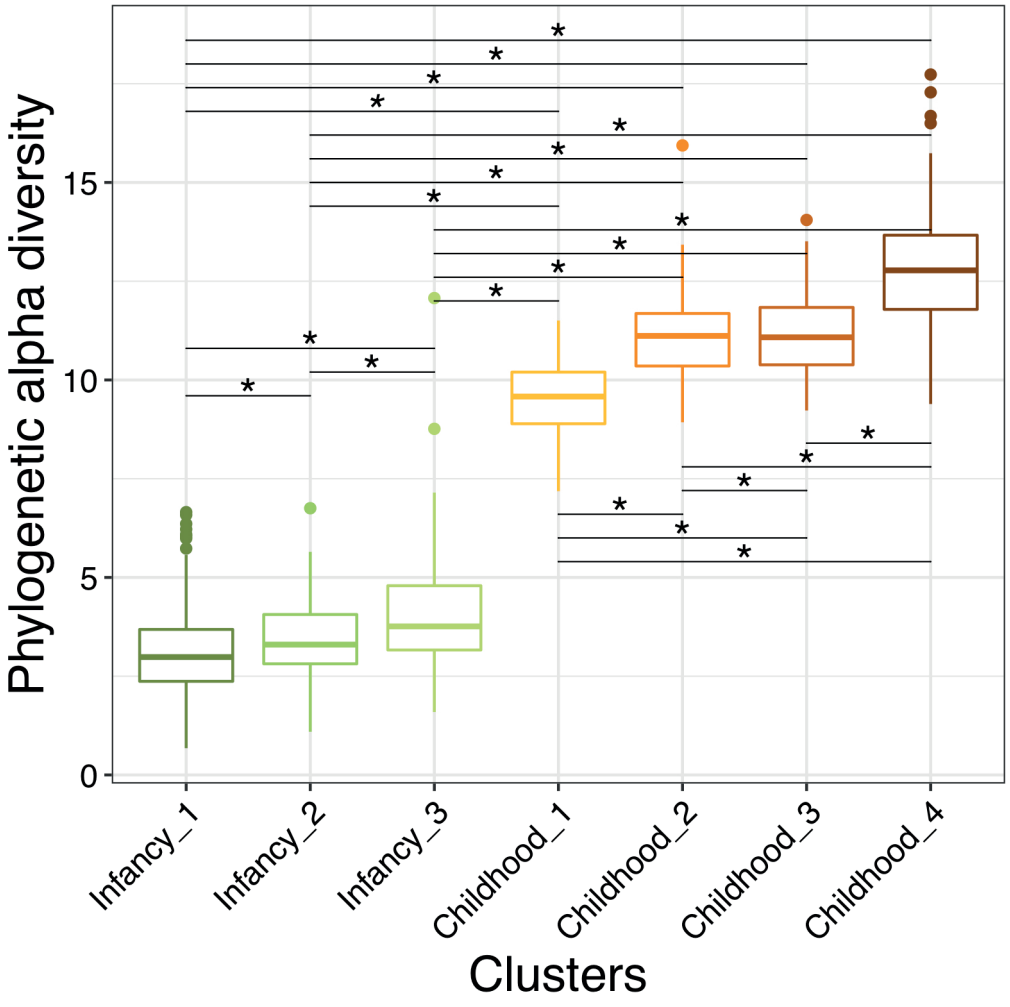


Figure S1. Box plots indicating phylogenetic diversity in the different microbial clusters. Diversity indices were calculated based on amplicon sequence variants in each microbial cluster. The boxes range from 25th to 75th percentiles, with center lines indicating medians. Outliers are displayed as points. Asterisks indicate p values < 0.05 (with FDR correction).

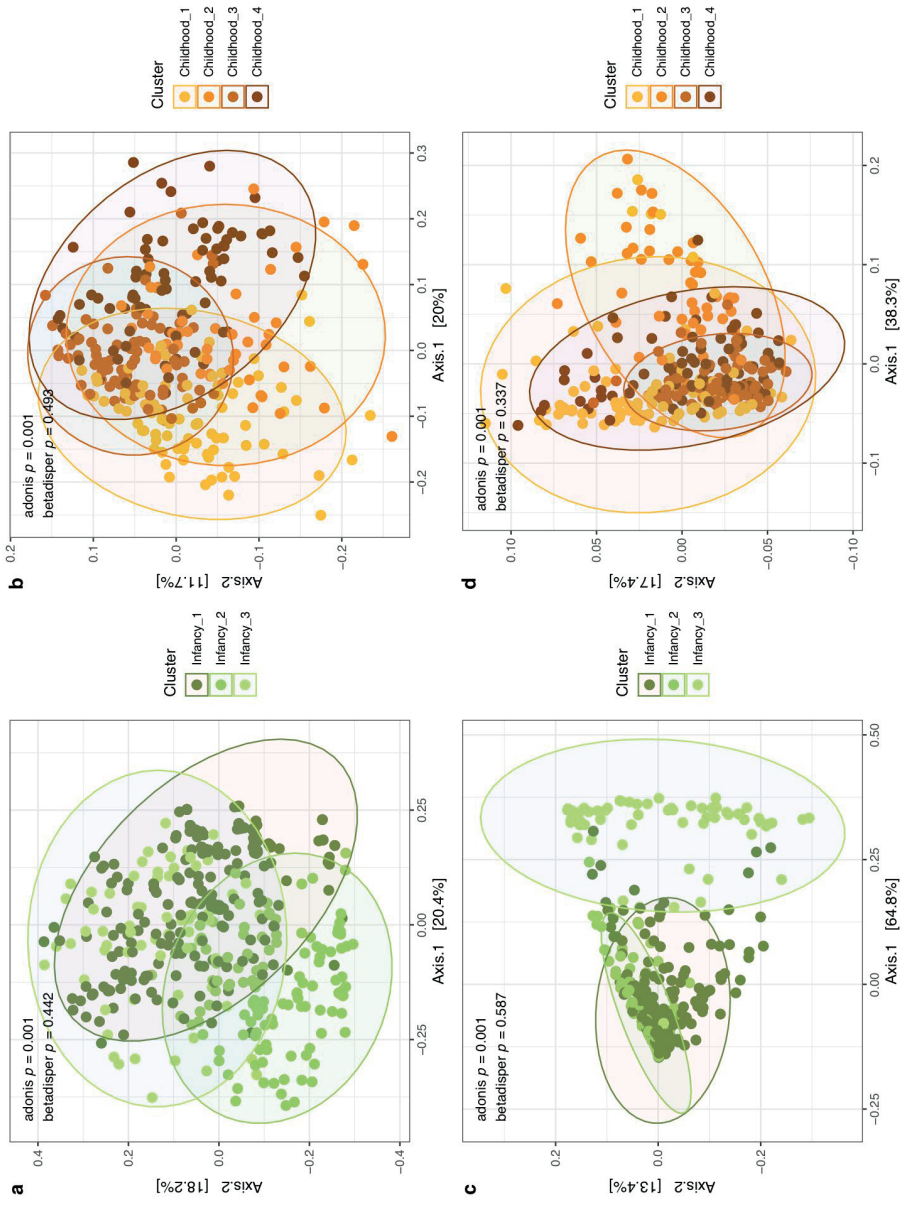
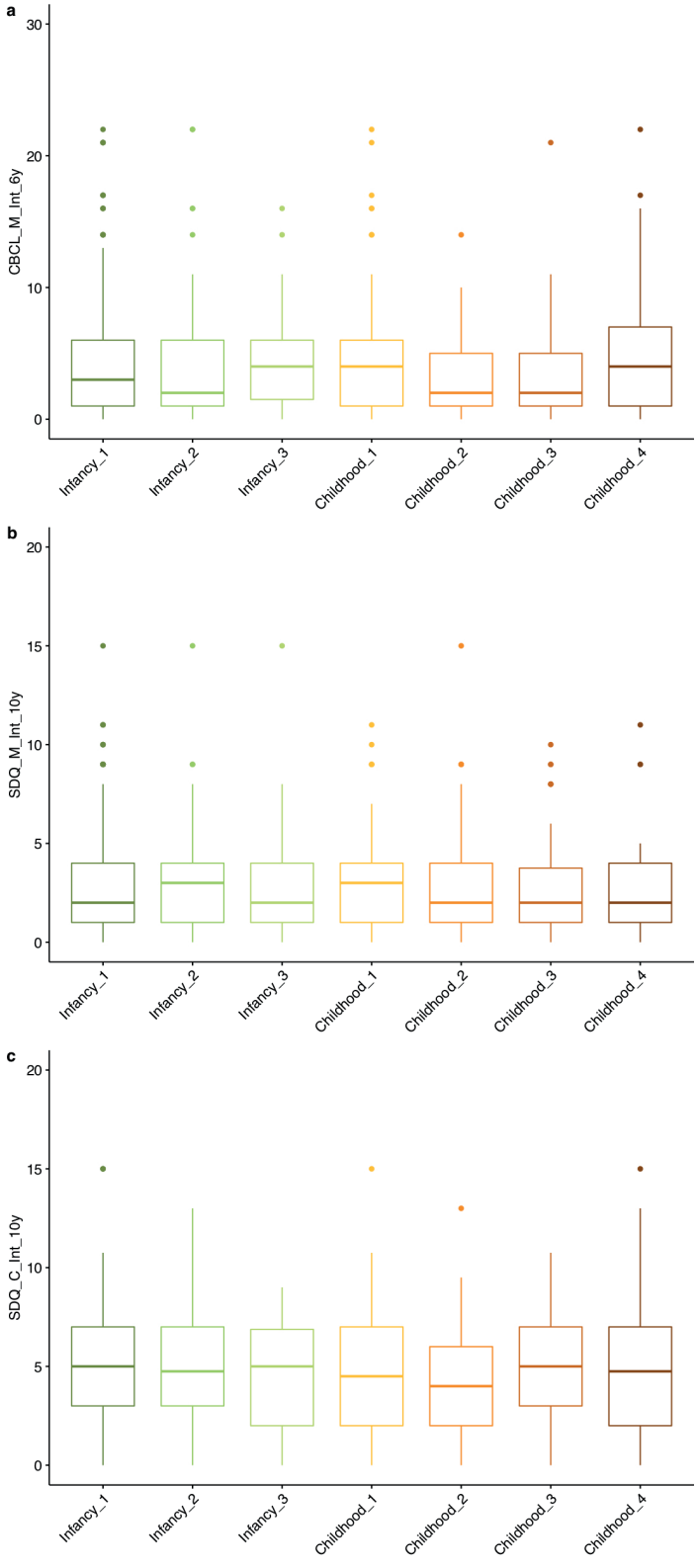


Figure S2. Beta diversity between microbial clusters. (a-b) Beta diversity was calculated from unweighted Unifrac distance obtained from relative abundance data at the genus level. (c-d) Beta diversity was computed from weighted Unifrac distance obtained from genera relative abundance data. Adonis reflects the significance of comparisons (N=1000 permutations). Betadisper refers to the significance of the homogeneity of variances of which a value higher than 0.05 means variances are homogeneous (N=1000 permutations).


a
CBCL_M_Int_6y
30
20
10
0
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4
b
SDQ_M_Int_10y
20
15
10
5
0
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4
c
SDQ_C_Int_10y
20
15
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Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4

Figure S3. Differences of internalizing behavior between microbial clusters. CBCL_M_Int_6y: internalizing behavior measured by maternal CBCL at age six. SDQ_M_Int_10y: internalizing behavior measured by maternal SDQ at age ten. SDQ_C_Int_10y: internalizing behavior measured by child SDQ at age ten. Wilcoxon rank sum tests were conducted with FDR adjustment. No significant differences were observed in internalizing behavior between the clusters.

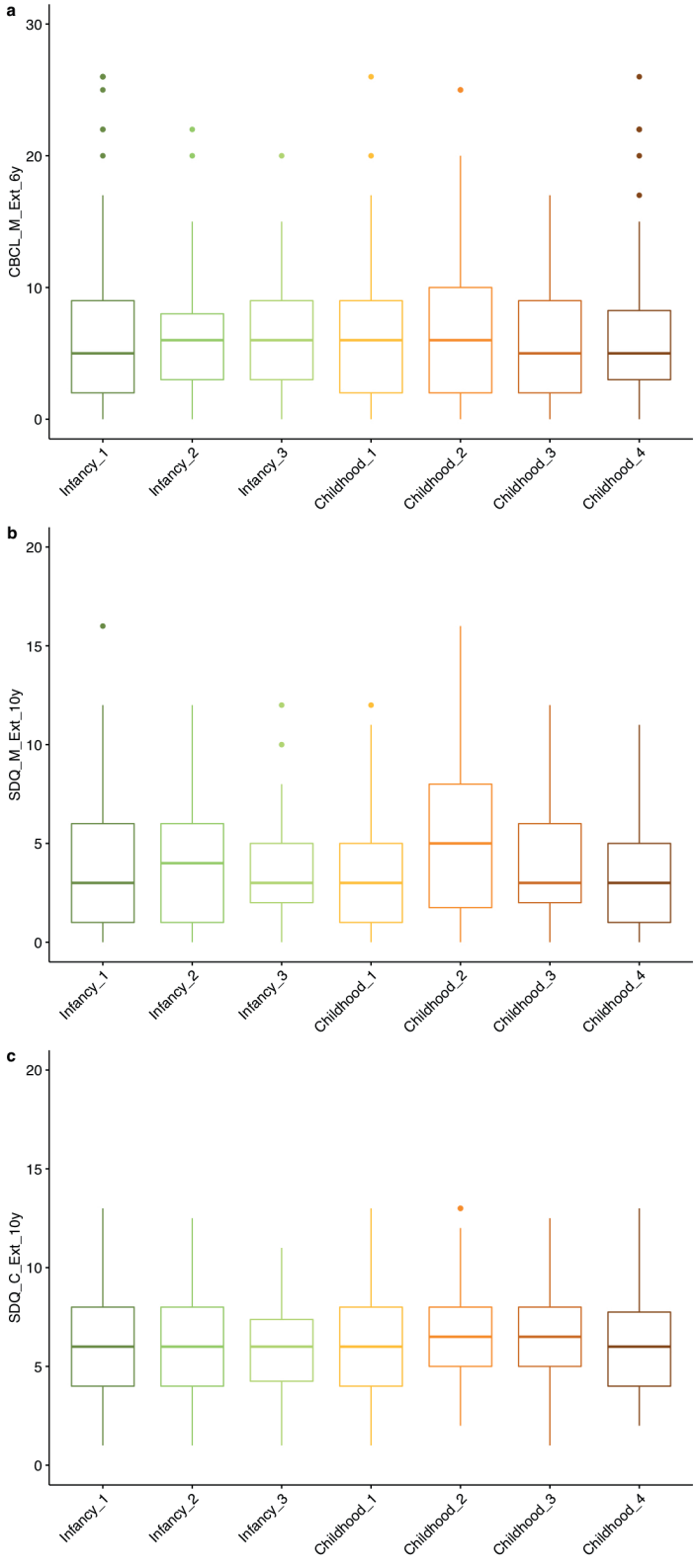
a
CBCL_M_Ext_6y
30
20
10
0
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4
b
SDQ_M_Ext_10y
20
15
10
5
0
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4
c
SDQ_C_Ext_10y
20
15
10
5
0
Infancy_1
Infancy_2
Infancy_3
Childhood_1
Childhood_2
Childhood_3
Childhood_4

Figure S4. Differences of externalizing behavior between microbial clusters. CBCL_M_Ext_6y: externalizing behavior measured by maternal CBCL at age six. SDQ_M_Ext_10y: externalizing behavior measured by maternal SDQ at age ten. SDQ_C_Ext_10y: externalizing behavior measured by child SDQ at age ten. Wilcoxon rank sum tests were conducted with FDR adjustment. No significant differences were observed in externalizing behavior between the clusters.

Table S1. The combination of infant DMM clusters in different runnings.

Running	Cluster	Number
1	V1	255
1	V2	127
1	V3	65
2	V1	255
2	V2	127
2	V3	65
3	**V1**	**256**
3	**V2**	**126**
3	**V3**	**65**
4	**V1**	**256**
4	**V2**	**126**
4	**V3**	**65**
5	V1	252
5	V2	125
5	V3	64
5	V4	6
6	**V1**	**256**
6	**V2**	**126**
6	**V3**	**65**
7	**V1**	**256**
7	**V2**	**126**
7	**V3**	**65**
8	V1	251
8	V2	132
8	V3	64
9	**V1**	**256**
9	**V2**	**126**
9	**V3**	**65**
10	V1	252
10	V2	125
10	V3	63
10	V4	7

The cluster combination in bold appeared the most frequently and was chosen in the study.

Table S2. The combination of childhood DMM clusters in different runnings.

Running	Cluster	Number
1	V1	86
1	V2	81
1	V3	67
1	V4	58
2	**V1**	**85**
2	**V2**	**79**
2	**V3**	**77**
2	**V4**	**51**
3	**V1**	**85**
3	**V2**	**79**
3	**V3**	**77**
3	**V4**	**51**
4	V1	78
4	V2	76
4	V3	56
4	V4	49
4	V5	33
5	V1	89
5	V2	80
5	V3	68
5	V4	55
6	**V1**	**85**
6	**V2**	**79**
6	**V3**	**77**
6	**V4**	**51**
7	**V1**	**85**
7	**V2**	**79**
7	**V3**	**77**
7	**V4**	**51**
8	**V1**	**85**
8	**V2**	**79**
8	**V3**	**77**
8	**V4**	**51**
9	**V1**	**85**
9	**V2**	**79**
9	**V3**	**77**
9	**V4**	**51**
10	**V1**	**85**

10	**V_2**	**79**
10	**V_3**	**77**
10	**V_4**	**51**

The cluster combination in bold appeared the most frequently and was chosen in the study.

Table S3. Significantly different KEGG orthologs with relative abundances higher than 0.5% between infant microbial clusters.

KEGG ortholog name	Average (%)	Infancy_1	Infancy_2	Infancy_3	Kruskal-Wallis test *p.adj*	Wilcoxon rank sum test *p.adj*		
						Infancy_1 *vs* Infancy_2	Infancy_1 *vs* Infancy_3	Infancy_2 *vs* Infancy_3
LDH, ldh; L-lactate dehydrogenase [EC:1.1.1.27]	0.53 ± 0.15	0.56 ± 0.11	0.58 ± 0.1	0.32 ± 0.17	<0.001	0.843	<0.001	<0.001
fabG; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]	0.65 ± 0.12	0.66 ± 0.11	0.61 ± 0.11	0.69 ± 0.13	<0.001	0.002	0.051	<0.001
IMPDH, guaB; IMP dehydrogenase [EC:1.1.1.205]	0.51 ± 0.14	0.55 ± 0.1	0.55 ± 0.1	0.25 ± 0.07	<0.001	0.598	<0.001	<0.001
glk; glucokinase [EC:2.7.1.2]	0.58 ± 0.23	0.64 ± 0.19	0.64 ± 0.2	0.25 ± 0.07	<0.001	0.961	<0.001	<0.001
recG; ATP-dependent DNA helicase RecG [EC:3.6.4.12]	1.01 ± 0.41	1.15 ± 0.31	1.08 ± 0.34	0.32 ± 0.16	<0.001	0.237	<0.001	<0.001
uvrD, pcrA; DNA helicase II / ATP-dependent DNA helicase PcrA [EC:3.6.4.12]	0.79 ± 0.25	0.84 ± 0.22	0.87 ± 0.14	0.43 ± 0.21	<0.001	0.891	<0.001	<0.001
dnaJ; molecular chaperone DnaJ	0.51 ± 0.14	0.55 ± 0.1	0.55 ± 0.09	0.26 ± 0.08	<0.001	0.843	<0.001	<0.001
cspA; cold shock protein (beta-ribbon, CspA family)	0.6 ± 0.14	0.58 ± 0.1	0.64 ± 0.1	0.57 ± 0.25	<0.001	<0.001	0.051	<0.001
K06999; phospholipase/carboxylesterase	0.53 ± 0.27	0.61 ± 0.22	0.58 ± 0.23	0.09 ± 0.08	<0.001	0.531	<0.001	<0.001
SPP; sucrose-6-phosphatase [EC:3.1.3.24]	1.42 ± 0.45	1.44 ± 0.43	1.3 ± 0.28	1.57 ± 0.66	0.006	0.016	0.243	0.014
dinJ; DNA-damage-inducible protein J	0.75 ± 0.33	0.85 ± 0.25	0.83 ± 0.26	0.19 ± 0.16	<0.001	0.843	<0.001	<0.001
K07496; putative transposase	0.58 ± 0.38	0.66 ± 0.34	0.68 ± 0.34	0.08 ± 0.13	<0.001	0.843	<0.001	<0.001

bgaB, lacA; beta-galactosidase [EC:3.2.1.23]	0.6 ± 0.24	0.68 ± 0.17	0.67 ± 0.19	0.19 ± 0.16	<0.001	0.925	<0.001	<0.001
ecfA2; energy-coupling factor transport system ATP-binding protein [EC:3.6.3.-]	0.59 ± 0.14	0.59 ± 0.13	0.63 ± 0.09	0.49 ± 0.23	<0.001	0.025	<0.001	<0.001

Table S4. Significantly different KEGG orthologs with relative abundances higher than 0.5% between childhood microbial clusters.

KEGG ortholog name	Average (%)	Childhood_1	Childhood_2	Childhood_3	Childhood_4	Kruskal-Wallis test *p.adj*	Wilcoxon rank sum test *p.adj*					
							Childhood_1 *vs* Childhood_2	Childhood_1 *vs* Childhood_3	Childhood_1 *vs* Childhood_4	Childhood_2 *vs* Childhood_3	Childhood_2 *vs* Childhood_4	Childhood_3 *vs* Childhood_4
fabG; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]	0.55 ± 0.05	0.51 ± 0.04	0.57 ± 0.03	0.56 ± 0.03	0.58 ± 0.05	<0.001	<0.001	<0.001	<0.001	0.1	0.342	0.008
DNMT1, dcm; DNA (cytosine-5)-methyltransferase 1 [EC:2.1.1.37]	0.8 ± 0.1	0.85 ± 0.1	0.78 ± 0.06	0.82 ± 0.08	0.74 ± 0.11	<0.001	<0.001	0.014	<0.001	0.001	0.131	<0.001
E2.2.1.1, tktA, tktB; transketolase [EC:2.2.1.1]	0.8 ± 0.09	0.85 ± 0.1	0.72 ± 0.08	0.82 ± 0.06	0.78 ± 0.08	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.003
pfkA, PFK; 6-phosphofructokinase 1 [EC:2.7.1.11]	0.55 ± 0.05	0.55 ± 0.06	0.52 ± 0.05	0.58 ± 0.03	0.52 ± 0.05	<0.001	<0.001	0.004	<0.001	<0.001	0.521	<0.001
xylB, XYLB; xylulokinase [EC:2.7.1.17]	0.53 ± 0.12	0.61 ± 0.13	0.44 ± 0.09	0.54 ± 0.07	0.48 ± 0.11	<0.001	<0.001	<0.001	<0.001	<0.001	0.047	<0.001
lacZ; beta-galactosidase [EC:3.2.1.23]	0.56 ± 0.1	0.58 ± 0.11	0.58 ± 0.07	0.57 ± 0.09	0.54 ± 0.11	0.001	0.23	0.78	0.008	0.331	<0.001	0.003
uvrD, pcrA; DNA helicase II / ATP-dependent DNA helicase PcrA [EC:3.6.4.12]	0.57 ± 0.03	0.57 ± 0.04	0.58 ± 0.02	0.56 ± 0.03	0.57 ± 0.04	<0.001	0.064	0.003	0.332	<0.001	0.021	0.033
pflA, pflC, pflE; pyruvate formate lyase activating	0.55 ±	0.57 ±	0.53 ±	0.56 ±	0.54 ±	<0.001	<0.001	0.021	<0.001	<0.001	0.125	0.002

enzyme [EC:1.97.1.4]	0.05	0.05	0.04	0.03	0.04							
feoA; ferrous iron transport protein A	0.54 ± 0.07	0.55 ± 0.07	0.47 ± 0.09	0.58 ± 0.03	0.54 ± 0.05	<0.001	<0.001	0.003	0.209	<0.001	<0.001	<0.001
feoB; ferrous iron transport protein B	0.55 ± 0.04	0.55 ± 0.04	0.53 ± 0.04	0.57 ± 0.03	0.54 ± 0.04	<0.001	0.011	<0.001	0.139	<0.001	0.227	<0.001
bglX; beta-glucosidase [EC:3.2.1.21]	0.67 ± 0.16	0.67 ± 0.16	0.84 ± 0.17	0.62 ± 0.07	6 ± 0.16	<0.001	<0.001	0.058	0.008	<0.001	<0.001	0.288
SPP; sucrose-6-phosphatase [EC:3.1.3.24]	0.94 ± 0.09	0.99 ± 0.09	0.88 ± 0.1	0.97 ± 0.06	0.91 ± 0.06	<0.001	<0.001	0.018	<0.001	<0.001	0.125	<0.001
K13653; AraC family transcriptional regulator	0.54 ± 0.12	0.6 ± 0.13	0.45 ± 0.09	0.56 ± 0.08	5 ± 0.11	<0.001	<0.001	0.003	<0.001	<0.001	0.019	<0.001

Table S5. Population characteristics of the samples in infancy.

	Infancy_1	Infancy_2	Infancy_3	Total	FDR-adjusted *p* value
Categorical variables					Fisher's test (excluding no record item)
Sample size					
Total	256	126	65	447	
1m	113	17	30	160	
	44.1 %	13.5 %	46.2 %	35.8 %	
3m	77	48	20	145	
	30.1 %	38.1 %	30.8 %	32.4 %	< 0.001
4m	66	61	15	142	
	25.8 %	48.4 %	23.1 %	31.8 %	
Delivery mode					
Not assisted vaginal delivery	225	106	42	373	
	87.9 %	84.1 %	64.6 %	83.4 %	
Assisted vaginal delivery	14	9	9	32	0.001
	5.5 %	7.1 %	13.8 %	7.2 %	
C section	8	9	11	28	
	3.1 %	7.1 %	16.9 %	6.3 %	
No record	9	2	3	14	
	3.5 %	1.6 %	4.6 %	3.1 %	
Child sex					
female	114	62	28	204	
	44.5 %	49.2 %	43.1 %	45.6 %	0.699
male	142	64	37	243	
	55.5 %	50.8 %	56.9 %	54.4 %	
Sibling(s) at birth					
Yes	152	79	22	253	
	59.4 %	62.7 %	33.8 %	56.6 %	0.001
No	97	43	41	181	
	37.9 %	34.1 %	63.1 %	40.5 %	
	7	4	2	13	

No record	2.7 %	3.2 %	3.1 %	2.9 %	
Solid food					
Yes	0	1	0	1	0.634
	0 %	0.8 %	0 %	0.2 %	
No	227	108	57	392	
	88.7 %	85.7 %	87.7 %	87.7 %	
No record	29	17	8	54	
	11.3 %	13.5 %	12.3 %	12.1 %	
Pet(s)					
Yes	33	17	11	61	0.703
	12.9 %	13.5 %	16.9 %	13.6 %	
No	207	103	51	361	
	80.9 %	81.7 %	78.5 %	80.8 %	
No record	16	6	3	25	
	6.2 %	4.8 %	4.6 %	5.6 %	
Gastroenteritis					
Yes	2	2	1	5	0.699
	0.8 %	1.6 %	1.5 %	1.1 %	
No	242	115	63	420	
	94.5 %	91.3 %	96.9 %	94 %	
No record	12	9	1	22	
	4.7 %	7.1 %	1.5 %	4.9 %	
Vomit					
Yes	43	12	8	63	0.327
	16.8 %	9.5 %	12.3 %	14.1 %	
No	201	105	56	362	
	78.5 %	83.3 %	86.2 %	81 %	
No record	12	9	1	22	
	4.7 %	7.1 %	1.5 %	4.9 %	
Diarrhea					
Yes	17	11	4	32	0.699
	6.6 %	8.7 %	6.2 %	7.2 %	
No	227	106	60	393	
	88.7 %	84.1 %	92.3 %	87.9 %	
	12	9	1	22	

No record	4.7 %	7.1 %	1.5 %	4.9 %	
Other diseases					
General	6	3	1	10	
	2.3 %	2.4 %	1.5 %	2.2 %	
Blood	1	0	0	1	
	0.4 %	0 %	0 %	0.2 %	
Digestive	6	6	2	14	
	2.3 %	4.8 %	3.1 %	3.1 %	
Locomotor	6	2	4	12	
	2.3 %	1.6 %	6.2 %	2.7 %	
Respiratory	3	5	0	8	0.444*
	1.2 %	4 %	0 %	1.8 %	
Skin	2	1	1	4	
	0.8 %	0.8 %	1.5 %	0.9 %	
Metabolic	0	1	1	2	
	0 %	0.8 %	1.5 %	0.4 %	
No	220	99	55	374	
	85.9 %	78.6 %	84.6 %	83.7 %	
No record	12	9	1	22	
	4.7 %	7.1 %	1.5 %	4.9 %	
Antibiotics					
Yes	0	5	0	5	
	0 %	4 %	0 %	1.1 %	
No	197	95	54	346	0.012
	77 %	75.4 %	83.1 %	77.4 %	
No record	59	26	11	96	
	23 %	20.6 %	16.9 %	21.5 %	
Other medications					
Medications for asthma	1	1	0	2	
	0.4 %	0.8 %	0 %	0.4 %	
Medications for eczema	1	3	0	4	
	0.4 %	2.4 %	0 %	0.9 %	0.327
Other systemic	17	3	7	27	
	6.6 %	2.4 %	10.8 %	6 %	

medications					
Other medications working locally	2 0.8 %	1 0.8 %	0 0 %	3 0.7 %	
No	223 87.1 %	109 86.5 %	57 87.7 %	389 87 %	
No record	12 4.7 %	9 7.1 %	1 1.5 %	22 4.9 %	
Numeric variables					Kruskal-Wallis test
Birth weight (g)	3594.5 ± 469.4 (N = 250, 97.7%)	3673.2 ± 488.4 (N = 126, 100%)	3642.5 ± 366.9 (N = 65, 100%)	3624.1 ± 461.8 (N = 441, 98.7%)	0.73
Maternal age (years)	32.2 ± 3.7 (N = 246, 96.1%)	33.3 ± 2.9 (N = 118, 93.7%)	32.5 ± 3.1 (N = 62, 95.4%)	32.6 ± 3.4 (N = 426, 95.3%)	0.075
Gestational age (weeks)	40.1 ± 1.2 (N = 256, 100%)	40.4 ± 1.2 (N = 126, 100%)	40 ± 1.2 (N = 65, 100%)	40.1 ± 1.2 (N = 447, 100%)	0.089
Breastfeeding	0.9 ± 0.3 (N = 242, 94.5%)	0.5 ± 0.4 (N = 120, 95.2%)	0.8 ± 0.4 (N = 62, 95.4%)	0.7 ± 0.4 (N = 424, 94.9%)	<0.001
CBCL_M_6y_Int	4.3 ± 4.5 (N = 223, 87.1%)	3.9 ± 4.4 (N = 106, 84.1%)	4 ± 3.6 (N = 51, 78.5%)	4.1 ± 4.3 (N = 380, 85%)	0.73
CBCL_M_6y_Ext	6.5 ± 5.3 (N = 223, 87.1%)	6 ± 4.3 (N = 106, 84.1%)	6.5 ± 4.5 (N = 51, 78.5%)	6.3 ± 4.9 (N = 380, 85%)	0.823
SDQ_M_10y_Int	2.9 ± 2.8 (N = 212, 82.8%)	3 ± 2.5 (N = 104, 82.5%)	2.7 ± 2.7 (N = 49, 75.4%)	2.9 ± 2.7 (N = 365, 81.7%)	0.73
SDQ_M_10y_Ext	3.8 ± 3.3 (N = 212, 82.8%)	4.2 ± 3.3 (N = 104, 82.5%)	3.7 ± 2.6 (N = 49, 75.4%)	3.9 ± 3.2 (N = 365, 81.7%)	0.73
SDQ_C_10y_Int	5 ± 2.9 (N = 221, 86.3%)	5 ± 3 (N = 106, 84.1%)	4.4 ± 2.5 (N = 47, 72.3%)	5 ± 2.9 (N = 374, 83.7%)	0.73
SDQ_C_10y_Ext	6.3 ± 2.7 (N = 221, 86.3%)	6.4 ± 2.8 (N = 106, 84.1%)	6 ± 2.3 (N = 47, 72.3%)	6.3 ± 2.7 (N = 374, 83.7%)	0.773

* The comparison was performed between the item "No" with the sum of items "General" to "Metabolic".

Table S6. Population characteristics of the samples in childhood.

	Childhood_1	Childhood_2	Childhood_3	Childhood_4	Total	FDR-adjusted *p* value
Categorical variables						Fisher's test (excluding no record item)
Sample size						
Total	85	51	79	77	292	
6y	50	32	33	30	145	
	58.8 %	62.7 %	41.8 %	39 %	49.7 %	0.088
10y	35	19	46	47	147	
	41.2 %	37.3 %	58.2 %	61 %	50.3 %	
Delivery mode						
No assisted vaginal delivery	67	41	67	66	241	
	78.8 %	80.4 %	84.8 %	85.7 %	82.5 %	
Assisted vaginal delivery	7	3	6	7	23	0.968
	8.2 %	5.9 %	7.6 %	9.1 %	7.9 %	
C section	8	3	2	3	16	
	9.4 %	5.9 %	2.5 %	3.9 %	5.5 %	
No record	3	4	4	1	12	
	3.5 %	7.8 %	5.1 %	1.3 %	4.1 %	
Child sex						
female	37	20	29	50	136	
	43.5 %	39.2 %	36.7 %	64.9 %	46.6 %	0.041
male	48	31	50	27	156	
	56.5 %	60.8 %	63.3 %	35.1 %	53.4 %	
Sibling(s) at birth						
Yes	46	32	34	43	155	
	54.1 %	62.7 %	43 %	55.8 %	53.1 %	
No	36	16	43	30	125	0.381
	42.4 %	31.4 %	54.4 %	39 %	42.8 %	
No record	3	3	2	4	12	
	3.5 %	5.9 %	2.5 %	5.2 %	4.1 %	
The time when solid						

food was first introduced						
≤26 weeks after birth	62 (22.19 ± 3.25)	42 (21.17 ± 2.88)	61 (22.39 ± 3.01)	56 (22.05 ± 3.32)	221 (22.02 ± 3.14)	0.381
	72.9 %	82.4 %	77.2 %	72.7 %	75.7 %	
>26 weeks after birth	17	4	6	6	33	
	20 %	7.8 %	7.6 %	7.8 %	11.3 %	
No record	6	5	12	15	38	
	7.1 %	9.8 %	15.2 %	19.5 %	13 %	
Pet(s)						
Yes	12	5	6	12	35	0.793
	14.1 %	9.8 %	7.6 %	15.6 %	12 %	
No	70	44	69	61	244	
	82.4 %	86.3 %	87.3 %	79.2 %	83.6 %	
No record	3	2	4	4	13	
	3.5 %	3.9 %	5.1 %	5.2 %	4.5 %	
If had antibiotics in the past one year						
Yes	7	4	9	9	29	0.968
	8.2 %	7.8 %	11.4 %	11.7 %	9.9 %	
No	72	43	69	62	246	
	84.7 %	84.3 %	87.3 %	80.5 %	84.2 %	
No record	6	4	1	6	17	
	7.1 %	7.8 %	1.3 %	7.8 %	5.8 %	
Other medications after birth for samples at age 6y						
Antihistamines						
≤5 times in the past one year	11	5	9	3	28	0.905
	22 %	15.6 %	27.3 %	10 %	19.3 %	
>5 times in the past one year	1	0	0	1	2	
	2 %	0 %	0 %	3.3 %	1.4 %	
No	36	23	22	23	104	
	72 %	71.9 %	66.7 %	76.7 %	71.7 %	
No record	2	4	2	3	11	

	4 %	12.5 %	6.1 %	10 %	7.6 %	
Non-antibiotic medications for respiratory diseases						
≤5 times in the past one year	13	7	8	8	36	
	26 %	21.9 %	24.2 %	26.7 %	24.8 %	
>5 times in the past one year	4	2	1	1	8	1
	8 %	6.2 %	3 %	3.3 %	5.5 %	
No	31	19	22	18	90	
	62 %	59.4 %	66.7 %	60 %	62.1 %	
No record	2	4	2	3	11	
	4 %	12.5 %	6.1 %	10 %	7.6 %	
Medications for skin						
≤5 times in the past one year	32	11	9	17	69	
	64 %	34.4 %	27.3 %	56.7 %	47.6 %	
>5 times in the past one year	4	3	8	3	18	0.1
	8 %	9.4 %	24.2 %	10 %	12.4 %	
No	12	14	14	7	47	
	24 %	43.8 %	42.4 %	23.3 %	32.4 %	
No record	2	4	2	3	11	
	4 %	12.5 %	6.1 %	10 %	7.6 %	
Other systemic medications						
≤5 times in the past one year	11	8	8	10	37	
	22 %	25 %	24.2 %	33.3 %	25.5 %	
>5 times in the past one year	3	1	0	1	5	0.968
	6 %	3.1 %	0 %	3.3 %	3.4 %	
No	34	19	23	16	92	
	68 %	59.4 %	69.7 %	53.3 %	63.4 %	
No record	2	4	2	3	11	
	4 %	12.5 %	6.1 %	10 %	7.6 %	
Other medications working locally						

≤5 times in the past one year	20	14	13	16	63	
	40 %	43.8 %	39.4 %	53.3 %	43.4 %	
>5 times in the past one year	4	0	2	2	8	0.905
	8 %	0 %	6.1 %	6.7 %	5.5 %	
No	24	14	16	9	63	
	48 %	43.8 %	48.5 %	30 %	43.4 %	
No record	2	4	2	3	11	
	4 %	12.5 %	6.1 %	10 %	7.6 %	
Others						
≤5 times in the past one year	1	0	1	0	2	
	2 %	0 %	3 %	0 %	1.4 %	
No	47	28	30	27	132	1
	94 %	87.5 %	90.9 %	90 %	91 %	
No record	2	4	2	3	11	
	4 %	12.5 %	6.1 %	10 %	7.6 %	
Other medications in the past one year for samples at age 10y						
Laxative						
Yes	4	1	4	5	14	
	11.4 %	5.3 %	8.7 %	10.6 %	9.5 %	
No	29	16	42	36	123	0.969
	82.9 %	84.2 %	91.3 %	76.6 %	83.7 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Diarrhea inhibitor						
Yes	1	1	1	1	4	
	2.9 %	5.3 %	2.2 %	2.1 %	2.7 %	
No	32	16	45	40	133	0.968
	91.4 %	84.2 %	97.8 %	85.1 %	90.5 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for ear infections						
Yes	4	0	1	1	6	0.487

	11.4 %	0 %	2.2 %	2.1 %	4.1 %	
No	29	17	45	40	131	
	82.9 %	89.5 %	97.8 %	85.1 %	89.1 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for strep throat and tonsillitis						
Yes	1	0	4	0	5	
	2.9 %	0 %	8.7 %	0 %	3.4 %	0.487
No	32	17	42	41	132	
	91.4 %	89.5 %	91.3 %	87.2 %	89.8 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for pneumonia						
No	33	17	46	41	137	Not applicable
	94.3 %	89.5 %	100 %	87.2 %	93.2 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for bronchitis						
Yes	1	0	1	0	2	
	2.9 %	0 %	2.2 %	0 %	1.4 %	0.968
No	32	17	45	41	135	
	91.4 %	89.5 %	97.8 %	87.2 %	91.8 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for paranasal sinuses						
Yes	1	0	0	0	1	
	2.9 %	0 %	0 %	0 %	0.7 %	0.793
No	32	17	46	41	136	
	91.4 %	89.5 %	100 %	87.2 %	92.5 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	

Medications for urinary tract infection						
No	33	17	46	41	137	Not applicable
	94.3 %	89.5 %	100 %	87.2 %	93.2 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for impetigo						
Yes	2	1	0	3	6	
	5.7 %	5.3 %	0 %	6.4 %	4.1 %	
No	31	16	46	38	131	0.549
	88.6 %	84.2 %	100 %	80.9 %	89.1 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Medications for mycosis						
Yes	3	1	1	0	5	
	8.6 %	5.3 %	2.2 %	0 %	3.4 %	
No	30	16	45	41	132	0.411
	85.7 %	84.2 %	97.8 %	87.2 %	89.8 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Others						
Yes	7	5	11	13	36	
	20 %	26.3 %	23.9 %	27.7 %	24.5 %	
No	26	12	35	28	101	0.968
	74.3 %	63.2 %	76.1 %	59.6 %	68.7 %	
No record	2	2	0	6	10	
	5.7 %	10.5 %	0 %	12.8 %	6.8 %	
Numeric variables						Kruskal-Wallis test
Birth weight (g)	3638.7 ± 456.8 (N = 84, 98.8%)	3562 ± 515.5 (N = 48, 94.1%)	3626.3 ± 491.2 (N = 79, 100%)	3596.6 ± 430.8 (N = 77, 100%)	3611.2 ± 468.3 (N = 288, 98.6%)	0.928
Maternal age (years)	32.6 ± 3.4 (N = 79, 92.9%)	33.3 ± 4.3 (N = 46, 90.2%)	32 ± 3.3 (N = 70, 88.6%)	32.6 ± 3.9 (N = 72, 93.5%)	32.6 ± 3.7 (N = 267, 91.4%)	0.753
Gestational age (weeks)	39.9 ± 1.1 (N = 85, 100%)	40 ± 1.1 (N = 51, 100%)	40.2 ± 1.4 (N = 79, 100%)	40 ± 1.4 (N = 77, 100%)	40 ± 1.3 (N = 292, 100%)	0.753
Breastfeeding	0.6 ± 0.4 (N = 83, 97.6%)	0.6 ± 0.4 (N = 49, 96.1%)	0.6 ± 0.4 (N = 75, 94.9%)	0.7 ± 0.4 (N = 73, 94.8%)	0.6 ± 0.4 (N = 280, 95.9%)	0.753

BMI	16.5 ± 2.1 (N = 81, 95.3%)	16.4 ± 2 (N = 49, 96.1%)	16.4 ± 1.5 (N = 78, 98.7%)	16.5 ± 1.7 (N = 70, 90.9%)	16.4 ± 1.8 (N = 278, 95.2%)	0.901
Diseases in the past one year						
General	2.4 ± 2.6 (N = 81, 95.3%)	1.4 ± 1.3 (N = 48, 94.1%)	1.7 ± 2.2 (N = 79, 100%)	1.6 ± 1.8 (N = 71, 92.2%)	1.8 ± 2.1 (N = 279, 95.5%)	0.571
Digestive	4.4 ± 4.9 (N = 81, 95.3%)	3.7 ± 4.5 (N = 48, 94.1%)	2.7 ± 2.7 (N = 79, 100%)	3.9 ± 4.6 (N = 71, 92.2%)	3.7 ± 4.3 (N = 279, 95.5%)	0.753
Respiratory	4.2 ± 3.7 (N = 81, 95.3%)	3.8 ± 3.4 (N = 49, 96.1%)	3.6 ± 3.7 (N = 79, 100%)	3.6 ± 3.1 (N = 71, 92.2%)	3.8 ± 3.5 (N = 280, 95.9%)	0.753
Skin	1.2 ± 3.4 (N = 81, 95.3%)	1.3 ± 3.4 (N = 48, 94.1%)	1.4 ± 3.1 (N = 78, 98.7%)	1.3 ± 3.3 (N = 70, 90.9%)	1.3 ± 3.3 (N = 277, 94.9%)	0.901
Total number of antibiotic treatments after birth	2.1 ± 2.7 (N = 42, 49.4%)	1.6 ± 1.7 (N = 32, 62.7%)	2.1 ± 2.8 (N = 51, 64.6%)	2.4 ± 2.1 (N = 31, 40.3%)	2 ± 2.4 (N = 156, 53.4%)	0.753
CBCL_M_6y _Int	4.7 ± 4.6 (N = 83, 97.6%)	3.4 ± 3.1 (N = 49, 96.1%)	3.5 ± 3.9 (N = 76, 96.2%)	4.9 ± 4.7 (N = 76, 98.7%)	4.2 ± 4.3 (N = 284, 97.3%)	0.571
CBCL_M_6y _Ext	6.3 ± 5.1 (N = 83, 97.6%)	7.1 ± 5.8 (N = 49, 96.1%)	6.3 ± 4.6 (N = 76, 96.2%)	6.6 ± 5.6 (N = 76, 98.7%)	6.5 ± 5.2 (N = 284, 97.3%)	0.928
SDQ_M_10y _Int	3.1 ± 2.6 (N = 83, 97.6%)	3.2 ± 3.1 (N = 48, 94.1%)	2.7 ± 2.4 (N = 74, 93.7%)	2.5 ± 2.3 (N = 72, 93.5%)	2.8 ± 2.6 (N = 277, 94.9%)	0.753
SDQ_M_10y _Ext	3.5 ± 3 (N = 83, 97.6%)	5.3 ± 4 (N = 48, 94.1%)	4.2 ± 3.1 (N = 74, 93.7%)	3.4 ± 2.8 (N = 72, 93.5%)	4 ± 3.2 (N = 277, 94.9%)	0.454
SDQ_C_10y_ Int	4.8 ± 3.1 (N = 81, 95.3%)	4.3 ± 2.6 (N = 47, 92.2%)	4.9 ± 2.6 (N = 78, 98.7%)	4.8 ± 3.1 (N = 76, 98.7%)	4.8 ± 2.9 (N = 282, 96.6%)	0.885
SDQ_C_10y_ Ext	6.2 ± 2.9 (N = 81, 95.3%)	6.6 ± 2.5 (N = 47, 92.2%)	6.5 ± 2.6 (N = 78, 98.7%)	6.2 ± 2.5 (N = 76, 98.7%)	6.4 ± 2.7 (N = 282, 96.6%)	0.885

Table S7. Food frequency outcomes for samples at age ten.

Item	Question	Childhood_1	Childhood_2	Childhood_3	Childhood_4	Total	*p*	*p.adj*
1	**Cooked vegetable (50g/day)**	1.6 ± 0.8 (N = 34, 97.1%)	1.7 ± 0.6 (N = 18, 94.7%)	1.6 ± 1.2 (N = 42, 91.3%)	1.9 ± 1.7 (N = 42, 89.4%)	1.7 ± 1.2 (N = 136, 92.5%)	0.50	0.90
2	**Raw vegetable (50g/day)**	0.7 ± 0.4 (N = 33, 94.3%)	0.8 ± 0.8 (N = 16, 84.2%)	0.9 ± 0.7 (N = 43, 93.5%)	0.8 ± 0.7 (N = 43, 91.5%)	0.8 ± 0.7 (N = 135, 91.8%)	0.83	0.90
3	**Fruit and vegetable juice (glass/day)**	0.4 ± 0.5 (N = 34, 97.1%)	0.4 ± 0.5 (N = 17, 89.5%)	0.8 ± 1.9 (N = 40, 87%)	0.3 ± 0.6 (N = 42, 89.4%)	0.5 ± 1.1 (N = 133, 90.5%)	0.13	0.83
4	**Mandarin (piece/day)**	0.2 ± 0.4 (N = 33, 94.3%)	0.1 ± 0.2 (N = 17, 89.5%)	0.3 ± 0.5 (N = 43, 93.5%)	0.3 ± 0.4 (N = 39, 83%)	0.2 ± 0.4 (N = 132, 89.8%)	0.41	0.90
5	**Other citrus fruit (piece/day)**	0.1 ± 0.1 (N = 32, 91.4%)	0 ± 0.1 (N = 16, 84.2%)	0.1 ± 0.1 (N = 42, 91.3%)	0 ± 0.1 (N = 46, 97.9%)	0.1 ± 0.1 (N = 136, 92.5%)	0.81	0.90
6	**Apple and pear (piece/day)**	0.6 ± 0.4 (N = 32, 91.4%)	0.7 ± 0.6 (N = 18, 94.7%)	0.9 ± 0.7 (N = 43, 93.5%)	0.7 ± 0.5 (N = 44, 93.6%)	0.7 ± 0.6 (N = 137, 93.2%)	0.67	0.90
7	**Banana (piece/day)**	0.2 ± 0.2 (N = 34, 97.1%)	0.3 ± 0.3 (N = 18, 94.7%)	0.2 ± 0.3 (N = 42, 91.3%)	0.3 ± 0.3 (N = 44, 93.6%)	0.3 ± 0.3 (N = 138, 93.9%)	0.67	0.90
8	**Other fruit (piece/day)**	0.3 ± 0.4 (N = 33, 94.3%)	0.5 ± 0.5 (N = 16, 84.2%)	0.3 ± 0.4 (N = 42, 91.3%)	0.4 ± 0.5 (N = 39, 83%)	0.4 ± 0.4 (N = 130, 88.4%)	0.59	0.90
9	**Apple sauce (tablespoon/day)**	0.3 ± 0.5 (N = 31, 88.6%)	0.2 ± 0.2 (N = 13, 68.4%)	0.3 ± 0.5 (N = 35, 76.1%)	0.4 ± 0.8 (N = 36, 76.6%)	0.3 ± 0.6 (N = 115, 78.2%)	0.90	0.90
10	**Total fruit (piece/day)**	1.3 ± 0.5 (N = 29, 82.9%)	1.5 ± 1 (N = 15, 78.9%)	1.7 ± 1.1 (N = 40, 87%)	1.7 ± 0.8 (N = 36, 76.6%)	1.6 ± 0.9 (N = 120, 81.6%)	0.15	0.83
11	**Total vegetable (50g/day)**	2.3 ± 1 (N = 33, 94.3%)	2.5 ± 1.1 (N = 16, 84.2%)	2.4 ± 1.4 (N = 42, 91.3%)	2.8 ± 2 (N = 42, 89.4%)	2.5 ± 1.5 (N = 133, 90.5%)	0.78	0.90

Total fruit is the sum of items 4 to 8. Total vegetable is the sum of items 1 and 2. Considering item 9 was consumed in low volume, we did not sum it into total fruit. FDR adjustments were conducted for multiple Kruskal-Wallis tests.

Table S8. Simple effects of extrinsic factors on the gut microbiota in infancy.

	R2%	Adjusted R2%	*p* value	Number of individuals	Number of genera
1m					
Delivery mode	1.61	0.243	0.055		
Birth weight	0.679	0	0.467		
Breastfeeding	1.634	0.956	0.001*	147	94
Child sex	0.628	0	0.7		
3m					
Delivery mode	1.791	0.281	0.095		
Birth weight	0.855	0.098	0.186		
Breastfeeding	2.181	1.435	0.001*	133	127
Child sex	0.765	0.007	0.394		
4m					
Delivery mode	1.805	0.271	0.104		
Birth weight	0.948	0.18	0.106		
Breastfeeding	1.979	1.219	0.001*	131	81
Child sex	0.66	0	0.807		
1-4m					
Child age	1.193	0.952	0.001*		
Delivery mode	0.644	0.157	0.001*		
Birth weight	0.241	0	0.492	411	155
Breastfeeding	0.938	0.696	0.001*		
Child sex	0.266	0.023	0.114		

A total of 1000 permutation tests were conducted to determine significance. Asterisks indicate *p* value < 0.05.

Table S9. Simple effects of extrinsic factors on the gut microbiota in childhood.

	R2%	Adjusted R2%	*p* value	Number of individuals	Number of genera
6y					
Delivery mode	2.679	0.084	0.377		
Birth weight	1.17	0	0.664		
Breastfeeding	1.442	0.146	0.255		
Age of the first solid food introduction	0.973	0	0.944	78	158
Child sex	1.437	0.14	0.27		
Total number of antibiotic treatments	1.1	0	0.8		
If had antibiotic treatment in past one year	1.287	0	0.466		
10y					
Delivery mode	2.849	0	0.656		
Birth weight	1.538	0	0.412		
Breastfeeding	1.744	0.208	0.231		
Age of the first solid food introduction	1.444	0	0.542	66	168
Child sex	1.684	0.148	0.285		
Total number of antibiotic treatments	1.952	0.42	0.114		
If had antibiotic treatment in past one year	1.68	0.144	0.27		
6-10y					
Child age	1.569	0.875	0.001*		
Delivery mode	1.586	0.19	0.121		
Birth weight	0.597	0	0.834		
Breastfeeding	1.032	0.335	0.017*		
Age of the first solid food introduction	0.738	0.039	0.32	144	181
Child sex	0.864	0.166	0.083		
Total number of antibiotic treatments	0.77	0.072	0.24		
If had antibiotic treatment in past one year	0.874	0.176	0.073		

A total of 1000 permutation tests were conducted to determine significance. Asterisks indicate p value < 0.05.

Table S10. Variance in microbial composition in infancy explained by internalizing or externalizing behavior without accounting for extrinsic factors.

	Behavior	R2%	Adjusted R2%	*p* value	Number of individuals	Number of genera
1m						
CBCL_M_6y	INT	0.695	0	0.617	136	
	EXT	0.863	0.123	0.122		
SDQ_M_10y	INT	0.981	0.214	0.05	131	94
	EXT	0.84	0.071	0.221		
SDQ_C_10y	INT	0.642	0	0.906	132	
	EXT	0.782	0.019	0.335		
3m						
CBCL_M_6y	INT	0.725	0	0.713	122	
	EXT	0.702	0	0.822		
SDQ_M_10y	INT	0.873	0.019	0.37	118	127
	EXT	0.863	0.009	0.407		
SDQ_C_10y	INT	0.797	0	0.547	122	
	EXT	0.881	0.055	0.314		
4m						
CBCL_M_6y	INT	0.775	0	0.596	122	
	EXT	0.626	0	0.965		
SDQ_M_10y	INT	0.636	0	0.976	116	81
	EXT	0.812	0	0.57		
SDQ_C_10y	INT	0.602	0	0.988	120	
	EXT	0.732	0	0.751		
1-4m						
CBCL_M_6y	INT	0.278	0.014	0.211	380	
	EXT	0.286	0.022	0.144		
SDQ_M_10y	INT	0.3	0.025	0.117	365	155
	EXT	0.311	0.036	0.073		
SDQ_C_10y	INT	0.261	0	0.57	374	
	EXT	0.283	0.015	0.231		

SDQ_M_10y: maternal SDQ at age 10. SDQ_C_10y: child SDQ at age 10. CBCL_M_6y: maternal CBCL at age 6. Number of 1000 permutation tests were conducted to determine significance.

Table S11. Variance in microbial composition in childhood explained by internalizing or externalizing behavior without accounting for extrinsic factors.

	Behavior	R2%	Adjusted R2%	*p* value	Number of individuals	Number of genera
6y						
CBCL_M_6y	INT	0.681	0	0.524	143	158
	EXT	0.736	0.032	0.338		
SDQ_M_10y	INT	0.786	0.04	0.32	135	
	EXT	1.254	0.512	0.001*		
SDQ_C_10y	INT	0.612	0	0.882	137	
	EXT	0.896	0.162	0.076		
10y						
CBCL_M_6y	INT	0.509	0	0.99	141	168
	EXT	0.588	0	0.893		
SDQ_M_10y	INT	0.769	0.061	0.253	142	
	EXT	0.839	0.131	0.128		
SDQ_C_10y	INT	0.8	0.107	0.139	145	
	EXT	0.692	0	0.445		
6-10y						
CBCL_M_6y	INT	0.328	0	0.687	284	181
	EXT	0.347	0	0.491		
SDQ_M_10y	INT	0.485	0.123	0.011*	277	
	EXT	0.62	0.258	0.002*		
SDQ_C_10y	INT	0.346	0	0.546	282	
	EXT	0.391	0.035	0.176		

SDQ_M_10y: maternal SDQ at age 10. SDQ_C_10y: child SDQ at age 10. CBCL_M_6y: maternal CBCL at age 6. Number of 1000 permutation tests were conducted to determine significance. Asterisk indicates *p* value < 0.05.

Table S12. MLM models for internalizing and externalizing behavior assessed by child SDQ at age ten.

	SDQ_C_10y_Int			SDQ_C_10y_Ext		
Response variable	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
1-4m						
Phylogenetic diversity	0.017	[-0.124, 0.157]	0.817	0.095	[-0.048, 0.238]	0.208
Streptococcus	-0.057	[-0.212, 0.098]	0.481	0.022	[-0.137, 0.179]	0.795
Clostridium sensu stricto 1	-0.099	[-0.280, 0.081]	0.294	0.069	[-0.115, 0.254]	0.472
6-10y						
Phylogenetic diversity	0.122	[-0.083, 0.329]	0.275	0.06	[-0.138, 0.257]	0.575
Streptococcus	-0.126	[-0.386, 0.135]	0.375	-0.044	[-0.292, 0.205]	0.742
Clostridium sensu stricto 1	0.236	[0.015, 0.462]	0.055	0.142	[-0.071, 0.354]	0.221
Bacteroides	0.051	[-0.127, 0.229]	0.6	-0.08	[-0.250, 0.091]	0.388
Barnesiella	0.14	[-0.139, 0.420]	0.359	0.06	[-0.206, 0.327]	0.682
Prevotella 9	-0.5	[-0.948, -0.053]	0.042*	0.656	[0.222, 1.087]	0.006*
Alistipes	0.087	[-0.171, 0.344]	0.536	-0.144	[-0.391, 0.102]	0.285
Coprococcus 2	-0.061	[-0.329, 0.208]	0.676	0.245	[-0.010, 0.502]	0.082
Ruminococcaceae NK4A214 group	-0.084	[-0.311, 0.144]	0.496	0.044	[-0.172, 0.260]	0.705
Phascolarctobacterium	-0.203	[-0.514, 0.109]	0.232	0.481	[0.180, 0.781]	0.004*
Sutterella	-0.24	[-0.498, 0.010]	0.085	0.033	[-0.208, 0.275]	0.802
Akkermansia	-0.182	[-0.482, 0.122]	0.269	-0.093	[-0.382, 0.193]	0.554
[Eubacterium] ruminantium group	-0.135	[-0.366, 0.102]	0.289	0.079	[-0.144, 0.301]	0.513
[Eubacterium] xylanophilum group	0.051	[-0.168, 0.267]	0.666	-0.01	[-0.214, 0.199]	0.926
Ruminococcaceae UCG-002	0.003	[-0.212, 0.223]	0.983	0.059	[-0.147, 0.266]	0.602
Ruminococcaceae UCG-005	0.089	[-0.201, 0.382]	0.573	0.046	[-0.232, 0.325]	0.76

Asterisk indicates p value < 0.05.

Table S13. MLM models for internalizing and externalizing behavior assessed by maternal CBCL at age six.

	CBCL_M_6y_Int			CBCL_M_6y_Ext		
Response variable	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
1-4m						
Phylogenetic diversity	0.022	[-0.126, 0.170]	0.773	0.106	[-0.039, 0.252]	0.164
Streptococcus	0.023	[-0.139, 0.186]	0.785	-0.036	[-0.197, 0.125]	0.667
Clostridium sensu stricto *1*	-0.149	[-0.343, 0.046]	0.146	0.19	[-0.002, 0.381]	0.06
6-10y						
Phylogenetic diversity	-0.046	[-0.253, 0.161]	0.68	0.075	[-0.126, 0.272]	0.487
Streptococcus	0.064	[-0.190, 0.317]	0.642	-0.216	[-0.461, 0.033]	0.11
Clostridium sensu stricto 1	0.053	[-0.176, 0.282]	0.672	0.055	[-0.170, 0.278]	0.648
Bacteroides	0.072	[-0.101, 0.245]	0.444	0.007	[-0.160, 0.174]	0.935
Barnesiella	-0.015	[-0.294, 0.265]	0.919	0.023	[-0.251, 0.291]	0.878
Prevotella 9	-0.194	[-0.674, 0.286]	0.455	0.238	[-0.213, 0.689]	0.33
Alistipes	0.049	[-0.203, 0.304]	0.72	-0.02	[-0.266, 0.223]	0.88
Coprococcus 2	-0.285	[-0.544, -0.028]	0.047*	0.196	[-0.062, 0.448]	0.159
Ruminococcaceae NK4A214 group	-0.016	[-0.235, 0.205]	0.897	-0.004	[-0.224, 0.213]	0.972
Phascolarctobacterium	-0.11	[-0.439, 0.218]	0.536	0.168	[-0.142, 0.477]	0.317
Sutterella	-0.125	[-0.377, 0.129]	0.365	-0.027	[-0.273, 0.218]	0.839
Akkermansia	0.119	[-0.177, 0.413]	0.46	0.009	[-0.281, 0.298]	0.952
[Eubacterium] ruminantium group	-0.063	[-0.297, 0.173]	0.622	-0.004	[-0.238, 0.224]	0.972
[Eubacterium] xylanophilum group	-0.11	[-0.316, 0.099]	0.332	0.003	[-0.204, 0.208]	0.979
Ruminococcaceae UCG-002	-0.083	[-0.291, 0.125]	0.465	0.136	[-0.071, 0.337]	0.222
Ruminococcaceae UCG-005	-0.024	[-0.308, 0.262]	0.876	0.146	[-0.131, 0.419]	0.329

Asterisk indicates *p* value < 0.05.

Table S14. Number of individuals included in MLM models in infancy and childhood.

	Infancy				Childhood		
	1m	**3m**	**4m**	**1-4m**	**6y**	**10y**	**6-10y**
SDQ_M_10y	123	109	107	339	72	64	136
SDQ_C_10y	123	113	111	347	74	66	140
CBCL_M_6y	128	114	114	356	78	66	144

SDQ_M_10y: maternal SDQ at age 10. SDQ_C_10y: child SDQ at age 10. CBCL_M_6y: maternal CBCL at age 6.

Table S15. Prevalence of genera in individuals involved in MLM models.

	SDQ_M_10y		SDQ_C_10y		CBCL_M_6y	
	1-4m	**6-10y**	**1-4m**	**6-10y**	**1-4m**	**6-10y**
Streptococcus	0.92	0.73	0.92	0.72	0.92	0.72
Clostridium sensu stricto 1	0.21	0.83	0.2	0.84	0.21	0.83
Bacteroides	0.13	0.96	0.12	0.96	0.13	0.97
Akkermansia	0.06	0.58	0.06	0.59	0.06	0.58
Alistipes	0.01	0.78	0.01	0.79	0.01	0.79
Prevotella 9	0.01	0.4	0.01	0.4	0.01	0.4
Sutterella	0.01	0.44	0.01	0.45	0.01	0.44
Barnesiella	0	0.64	0	0.66	0	0.65
[Eubacterium] ruminantium group	0	0.26	0	0.27	0	0.28
Coprococcus 2	0	0.67	0	0.69	0	0.69
Ruminococcaceae NK4A214 group	0	0.59	0	0.59	0	0.59
Ruminococcaceae UCG-002	0	0.88	0	0.88	0	0.88
Ruminococcaceae UCG-005	0	0.59	0	0.61	0	0.6
Phascolarctobacterium	0	0.33	0	0.34	0	0.33
[Eubacterium] xylanophilum group	0	0.34	0	0.34	0	0.33

SDQ_M_10y: maternal SDQ at age 10. SDQ_C_10y: child SDQ at age 10. CBCL_M_6y: maternal CBCL at age 6.

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Chapter 4

Gut microbiota and child behavior in early puberty: does child gender play a role?

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Abstract

Background: A growing number of studies have indicated relations between the gut microbiota and mental health. However, to date, there is a scarcity of microbiota studies in community samples in early puberty. The current preregistered study (https://osf.io/wu2vt) investigated gut microbiota composition in relation to gender in low-risk children and explored behavioral associations with gut microbiota composition and metabolites in the same samples, together with the potential role of gender.

Results: Fecal microbiota composition was analyzed in twelve-year-old children (N=137) by 16S rRNA gene sequencing and quantitative PCR. Modest gender differences were observed in beta diversity. Bayesian models showed multiple behavioral relations to both relative and absolute abundances of individual taxa, including positive associations of *Ruminococcaceae* UCG-004 and *Parasutterella* with mother-reported internalizing behavior, and negative associations of *Odoribacter* and *Parasutterella* with mother-reported externalizing behavior and child-reported prosocial behavior, respectively. Additionally, *Prevotella* 9 was positively related to mother-reported externalizing behavior, confirming earlier findings on the same cohort at age ten years. Gender differences were observed in the relations of *Parasutterella*, *Coprococcus* 3, and *Ruminococcaceae* UCG-003 with internalizing, externalizing, and prosocial behavior, respectively. Limited behavioral relations were observed regarding fecal metabolites.

Conclusions: Our findings describe links between the gut microbiota and child behavior, together with differences between child genders in these relations, in low-risk early pubertal children. Importantly, this study confirmed earlier findings in this cohort of positive relations between *Prevotella* 9 and externalizing behavior at age ten years. Results also show the merit of including absolute abundances in microbiota studies.

Keywords: gut microbiota; microbiota-derived fecal metabolites; child behavior; child gender; puberty; *Parasutterella*; *Prevotella* 9; absolute abundance.

Introduction

The human gut is colonized by a great number of microorganisms that are collectively referred to as the gut microbiota. These microorganisms play a critical role in human health (Fan & Pedersen, 2021). Many factors can influence gut microbiota composition (Hasan & Yang, 2019). Biological gender is likely to affect the microbiota but has received limited attention in the past (Ding & Schloss, 2014; Falony et al., 2016; Jaggar, Rea, Spichak, Dinan, & Cryan, 2020; Manosso et al., 2021). Furthermore, a growing number of studies show that the gut microbiota can affect and be affected by brain functions along the microbiota-gut-brain axis (MGBA) (Cryan et al., 2019). However, to date only few studies have explored the gut microbiota and its relations to behavior in a low-risk community sample of children, especially in puberty. Therefore, the current study explored gender differences in gut microbiota composition in low-risk children at the onset of puberty, and then investigated potential associations between the gut microbiota and child behavior, taking child gender into account.

Population-level studies have reported that biological gender is moderately related to adult gut microbiota composition (Ding & Schloss, 2014; Falony et al., 2016). According to a large cohort study of the Flemish Gut Flora Project, gender was ranked as the tenth important factor among 69 covariates (Falony et al., 2016). Another, clustering-based, study of the Human Microbiome Project revealed that adult males were three times more likely to harbor a community type enriched in *Prevotella* but depleted in *Bacteroides*, in comparison with women (Ding & Schloss, 2014). Additionally, other adult studies showed gender-dependent differences in abundances of specific microbial taxa, such as *Prevotella*, *Bifidobacterium*, *Akkermansia*, and *Ruminococcus* (Mueller et al., 2006; Oki et al., 2016; Takagi et al., 2019). Although gender is commonly speculated to impact the gut microbiota from puberty on due to gonadal hormones, its association to microbiota composition has not been well explored in children at this age (Jaggar et al., 2020; Manosso et al., 2021; Valeri & Endres, 2021). A recent, albeit small, study found several differentially abundant microbial taxa between pubertal boys and girls, including higher relative abundances of *Alistipes* and *Parabacteroides* in girls (Yuan, Chen, Zhang, Lin, & Yang, 2020).

Despite the scarcity of studies in these low-risk populations, some associations between gut microbiota composition and child behavior have been reported in the past years. Earlier studies showed that increased alpha diversity was related to decreased cognitive ability, fear reactivity, and internalizing problems in infants and pre-schoolers (Aatsinki et al., 2019; Carlson et al., 2018; Laue et al., 2021; Van De Wouw et al., 2022). Regarding specific microbial taxa, the genus *Prevotella* appears to stand out. For example, Loughman et al. found that more *Prevotella* at age one predicted less subsequent internalizing behavior at age two (Loughman et al., 2020). Furthermore, we previously reported a positive relation of *Prevotella* 9 with externalizing behavior in middle childhood (Ou, Belzer, Smidt, & de Weerth, 2022). Notably, gender may likely affect associations between the gut microbiota and child behavior in puberty, as gender-specific differences in behavior at this age are often observed, such as in internalizing and externalizing behavioral problems (Chaplin & Aldao, 2013; Leadbeater, Kuperminc, Blatt, & Hertzog, 1999).

Specific microbiota-derived metabolites are often assumed to be important biological mediators in the complex bidirectional pathways of the MGBA (Cryan et al., 2019; Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019; Mirzaei et al., 2021; Simpson et al., 2021), despite the fact that the underlying mechanisms remain largely unknown. Among these microbial metabolites, short-chain fatty acids (SCFAs), the major colonic fermentation products of indigestible fiber, are thought to influence the communication along the MGBA through immune, endocrine, and vagal pathways (Dalile et al., 2019; Mirzaei et al., 2021). SCFAs, especially butyrate, have been associated with alleviated anxious and depressive symptoms in mental disorders (Dalile et al., 2019; Simpson et al., 2021). Regarding branched-chain fatty acids (BCFAs), increased fecal isobutyrate has been related to less internalizing behavior in pre-schoolers (Van De Wouw et al., 2022), while increased fecal isovalerate has been observed in depressed adults compared to neurotypical controls (Szczesniak, Hestad, Hanssen, & Rudi, 2016). Additionally, microbiota-derived lactate might lead to the increases in urine and blood lactate that are seen in depressed subjects; conversely, microbiota-generated lactate may also support hippocampal neurogenesis as a potential anti-depressant molecule (Ortega et al., 2022).

The present preregistered study (https://osf.io/wu2vt) was carried out on an ongoing longitudinal cohort of low-risk community children when they were 12 years old. The study had two aims: (1) to describe potential child gender-related differences in gut microbiota composition at the onset of puberty, and (2) to explore potential associations between gut microbiota composition (i.e., diversity and microbial taxon abundances), microbiota-derived fecal metabolites (i.e., SCFAs, BCFAs and lactate), and child behavioral measures (i.e., internalizing, externalizing, and prosocial behavior) at this age (Figure 1). Internalizing behavior refers to behavioral problems that influence internal psychological conditions, such as depression, anxiety, somatic states, and social withdrawal, whereas externalizing behavior is manifested as outward behavior, such as aggression, acting out, hyperactivity, hostility, and antisocial behavior. Prosocial behavior is interpreted as an intention to voluntarily help and benefit others. To obtain a panoramic view of the child's behavior, both self-report and maternal report data were collected. Given the scarcity of previous studies in low-risk pubertal children, we did not set up specific hypotheses on the associations regarding the second aim.

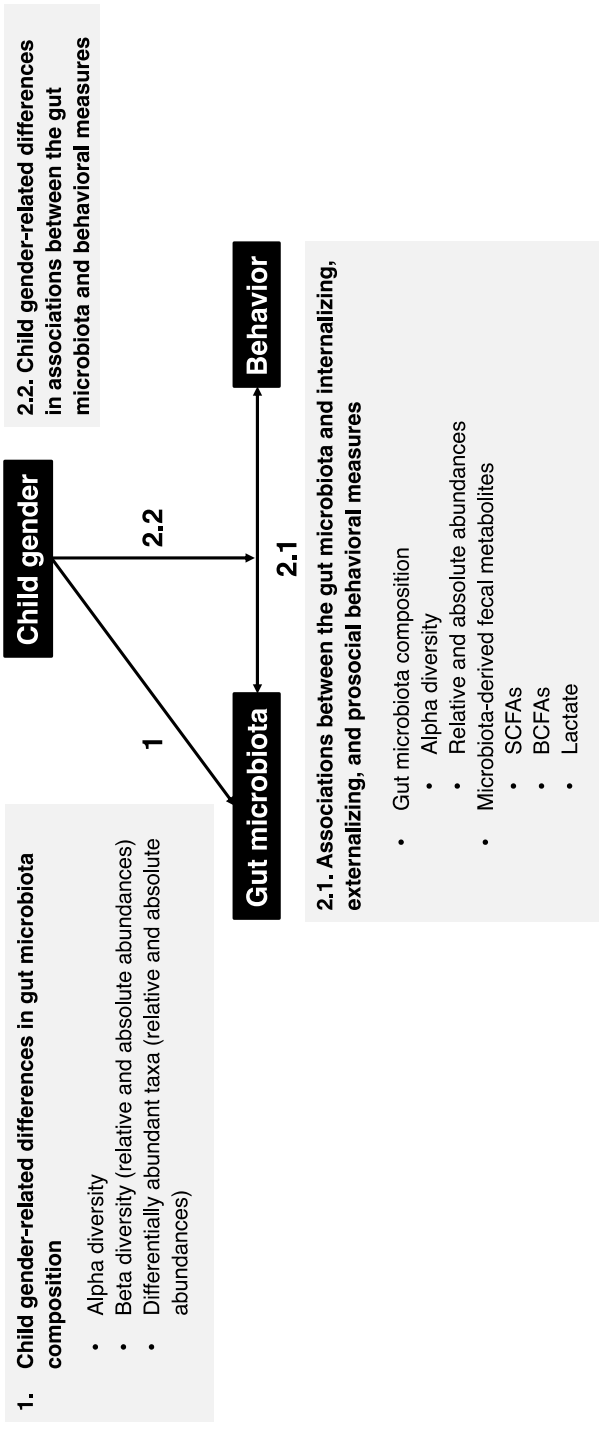

Figure 1. Main research questions of the study. SCFAs, short-chain fatty acids; BCFAs, branched-chain fatty acids.

Although relative abundance data are frequently and widely used in human studies to describe gut microbiota composition, it has been pointed out that such data come with inherent limitations, including high false discovery rates, more correlational biases, and the insufficiency to fully capture individual differences among samples (Barlow, Bogatyrev, & Ismagilov, 2020; Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020). Therefore, along with relative abundance data, we also included absolute abundance data in the current study.

Materials and Methods

Study subjects

The study consisted of low-risk children (N=137) aged around 12 years (12.7 ± 0.3) from the ongoing longitudinal Dutch study named BIBO (Basale Invloeden op de Baby Ontwikkeling; N=193 originally recruited in pregnancy) (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011), with approval from the ethical committee of the Faculty of Social Sciences of Radboud University (ECG300107, ECG13012012, SW2017-1303-497 and SW2017-1303-498). The original recruitment criteria and procedures are described elsewhere (Beijers et al., 2011). Characteristics of the current sample are presented in Table 1. The present study was preregistered on the Open Science Framework via this link https://osf.io/wu2vt.

Procedures of data collection

Child stool samples were collected in sterilized plastic tubes by either children or their parents immediately after defecation. Samples were temporarily kept in home freezers at -20°C until being delivered to the lab and stored at -80°C prior to being processed. Children as well as their mothers were asked to fill in online questionnaires separately by using personal links. In two cases, fathers filled in the questionnaire, as the mothers were unavailable during the data collection period. For an easy interpretation, they were still called maternal reports and included in the study. The questionnaires filled in by both children and mothers pertained behavior. Additionally, children filled in questionnaires about diet, and mother completed questionnaires about child health and demographics.

Measures

Gut microbiota composition

Briefly, DNA was extracted from 0.01 to 0.13 g of fecal samples by using the Maxwell 16 Total RNA system (Promega, Wisconsin, USA) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN), as described previously (Gu et al., 2018). The V4 region of 16S ribosomal RNA (rRNA) gene of bacteria and archaea was amplified for each sample in duplicate, then amplicons were purified and adjusted to 200 ng each sample before being sequenced, by following steps delineated earlier (Ou et al., 2022). Amplicon sequence variants (ASVs) were identified from 16S rRNA gene sequence data through *NG-Tax* 2.0 (Poncheewin et al., 2020; Ramiro-Garcia et al., 2018). ASVs were assigned to taxa referring to SILVA_132_SSU 16S rRNA gene reference database (Quast et al., 2012). Subsequently, we obtained a total of 32,081,185 reads with median reads of 226, 625 per sample.

To obtain total microbial abundances within individuals, qPCR reactions were performed in triplicate as follows: (1) 5 µl of SYBR Green Master Mix, 2.6 µl of nuclease-free water, 0.2 µl of 331F universal primer (5'-TCCTACGGGAGGCAGCAGT), 0.2 µl of 797R universal primer (5'-GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni, Martin, Jacques, & Hunter, 2002), and 2 µl of 1 ng/µl templates (in standard reactions, full-length 16S rRNA gene amplicons of *Escherichia coli*, diluted to 10^8 to 10^1 copy numbers/µl) were used for each reaction; (2) the qPCR program included 10 min of initial denaturation at 95 °C and 40 quantification cycles, consisting of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and elongation for 15 s at 72 °C. The raw data were then pre-processed by the CFX Maestro Software.

Fecal metabolites

The supernatant of the mixture of 0.2 g fecal sample and 800 µL demineralized water was treated with Carrez reagents to remove protein (Nyanga, Nout, Smid, Boekhout, & Zwietering, 2013; Selak et al., 2016). Then, the deproteinized supernatant was analyzed by high performance liquid chromatography (HPLC; Shimadzu LC-2030C Plus), equipped with refractive index and UV light (210 nm) detectors. The separation was completed on a Shodex SH1011 column with a flow rate of 1 mL/min at 45°C. The eluent was 0.01 N sulphuric acid.

Behavioral measures

To assess problem behavior (i.e., internalizing and externalizing behavior) and prosocial behavior, children and mothers were asked to fill in the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 1997). Higher scores on the internalizing and externalizing scales reflect difficulties, while higher scores on the prosocial scale indicate strengths.

Internal consistency of the scales was confirmed by ω_{total} estimates (Revelle & Condon, 2019), computed by the *psych* R package (Revelle, 2021). Most of the estimates were larger than 0.7 (Table S1), reflecting acceptable internal consistency and indicating that the scales were reliable. Only the child prosocial behavioral scale showed a questionable estimate, but this conformed to previous Dutch research (van Widenfelt, Goedhart, Treffers, & Goodman, 2003), and thus was included in the current study. Internalizing, externalizing, and prosocial behavioral scales were positively correlated between child and maternal reports with Spearman correlation coefficients of 0.55, 0.51, and 0.24, respectively (Figure S1).

Additional variables

In addition to the child gender (girl or boy) (Chaplin & Aldao, 2013; Fabes, Carlo, Kupanoff, & Laible, 1999), we considered the following variables as potential confounders (Bongers, Koot, van der Ende, & Verhulst, 2003; Falony et al., 2016; O'Neil et al., 2014), which may influence both the microbial predictor and the behavioral outcome: (1) child age in days (one missing value was identified among 137 samples, and replaced with the average value of the remaining available data); (2) two food factors (i.e., Factor 1: healthy foods; Factor 2: snacks) based on a 25-item food frequency questionnaire (Table S2), scored on a seven-point scale and collected during the online questionnaire fill-in procedure.

Besides, as a potential intermediate between diet and behavior (Monshouwer et al., 2006; Zhao, 2013), zBMI was calculated from child height, weight, child gender, and age

according to the WHO Growth Reference via the *zscore* R package (missing values were processed as described in Supplementary materials) (Myatt & Guevarra, 2019).

Additionally, three potential covariates of the gut microbiota (i.e., variables considered to only impact the microbial predictor) (Cryan et al., 2019; Derrien, Alvarez, & de Vos, 2019), were collected by a child health questionnaire (Beijers, Jansen, Riksen-Walraven, & De Weerth, 2010), during the online questionnaire fill-in procedure: Whether a child (1) took antibiotics, (2) had diarrhea, and (3) had constipation in the past one year. These variables were dummy-scored as no = 0 and yes = 1.

Statistical analyses

All analyses were performed in R studio (version 4.1.1; this version is an update of the version 3.6.1 that was described in the preregistration).

Gut microbiota data transformation

Both relative (0-100%) and absolute abundances (counts per gram of wet feces) at the genus level were calculated for each sample. Absolute abundance of a genus-level taxon in each sample was obtained by multiplying the relative abundance of this taxon by the total microbial abundance in this sample (Jian et al., 2020). The absolute abundance was then corrected for 16S rRNA gene copy-number variation referring to the rrnDB database (Stoddard, Smith, Hein, Roller, & Schmidt, 2015), by dividing this abundance value by the 16S rRNA gene copy number.

First aim: Child gender-related differences in gut microbiota composition

Alpha diversity (i.e., Chao1, Shannon, and phylogenetic diversity) was calculated based on ASV count data by using the *ape* and *picante* R packages (Paradis, 2020; W. Kembel, 2020), and was then compared between boys and girls by means of Wilcoxon rank sum tests with FDR adjustment.

Beta diversity was compared between genders by using different methods in calculating distance/dissimilarity matrices via the *vegan* (Oksanen, 2020) and *phyloseq* (McMurdie & Holmes, 2013) R packages. These methods included unweighted UniFrac, weighted UniFrac, unweighted Jaccard, Bray-Curtis, and Aitchison. Except for the Aitchison distance (i.e., The Euclidean distance based on clr-transformed count data), for the rest of the beta-diversity methods, we either transformed original ASV counts into relative abundances, or applied log transformation to absolute abundances beforehand. Then, we measured the univariate and cumulative variance (R^2%) in the gut microbiota explained by the additional variables and behavioral measures, by performing redundancy analysis (RDA).

Differentially abundant genus-level microbial taxa between genders were identified through Wilcoxon rank sum tests with FDR correction for both relative and absolute abundances.

Second aim: Associations between the gut microbiota and internalizing, externalizing, and prosocial behavioral measures

Through the *brms* (Bürkner, 2017) and *bayestestR* (Makowski, Ben-Shachar, & Lüdecke, 2019) R packages, we performed Bayesian linear regression models to analyze if each behavioral measure could be predicted by gut microbiota composition (i.e., alpha diversity, and relative

abundances and log-transformed absolute abundances both at the genus level) or microbiota-derived fecal metabolites (i.e., SCFAs, BCFAs, lactate, total SCFAs, total BCFAs, and the ratio of total BCFAs to total SCFAs). Numeric variables were standardized before being used in the models. To avoid over-sparsity and retain more microbial taxa, we performed the models on taxa prevalent in more than 10% of all subjects. Several different models were conducted as follows:

(1) **Model 0** $B_i \sim G_j$ was used to measure the independent relation between the outcome variable (B_i is the matrix of behavioral measures, with "*i*" indicating one measure assessed either by child or maternal reports) and the predictor (G_j is the matrix of alpha diversity, microbial abundances, and fecal metabolites, with "*j*" being a diversity parameter, a taxon, or a metabolite);

(2) According to the principals based on a causal diagram, namely the directed acyclic graph (Cinelli, Forney, & Pearl, 2020), we accounted for potential confounders in **Model 1** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2*;

(3) To remove the potential intermediate effect of zBMI between diet and behavior, we additionally included it in **Model 2** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2* + *zBMI*;

(4) Antibiotics, diarrhea, and constipation were regarded as covariates of microbial predictors only as described above, and thus were treated as neutral variables unnecessary to account for (Cinelli et al., 2020). However, since these variables have been included in other mental health related studies, we performed sensitivity analyses to test the consistency between our models by using **Model 3** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2* + *zBMI* + *Antibiotics* + *Diarrhea* + *Constipation*.

To explore child gender-related differences in the associations between the gut microbiota and behavioral measures, we added an additional interaction term of child gender and the predictor to the models displayed earlier:

(1) **Model 1 with the interaction** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2* + G_j*: child gender*;
(2) **Model 2 with the interaction** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2* + *zBMI* + G_j*: child gender*;
(3) **Model 3 with the interaction** $B_i \sim G_j$ + *child gender* + *child age* + *food Factor 1* + *food Factor 2* + *zBMI* + *Antibiotics* + *Diarrhea* + *Constipation* + G_j*: child gender*.

Significance

For non-multiple tests, a *p* value lower than 0.05 was defined as significant. The significance was corrected by the FDR method for multiple tests, with adjusted *p* lower than 0.1 accepted as significant. Regarding Bayesian models, parameter estimates were regarded as significant when the 95% credible intervals (CIs) excluded 0.

Results

Demographics and descriptives

Population demographics and descriptives are presented in Table 1. About 47% (64/137) of the study subjects were girls. Girls significantly showed more child-reported internalizing

difficulties and more mother-reported prosocial behavior than boys. No gender differences were observed in other behavioral measures and variables. Finally, child reports reflected significantly more internalizing and externalizing behavior than maternal reports.

Table 1. Population demographics and descriptives at the age of 12 years.

		Total N = 137	Girls N = 64	Boys N = 73	*p.adj*	
Numeric variable			Mean ± SD		Wilcoxon test Girl *vs* Boy	Child *vs* Maternal reports†
Age in days		4638 ± 115	4620 ± 109	4654 ± 118	0.165	-
Food Factor 1		0 ± 0.82	0.05 ± 0.83	-0.04 ± 0.81	0.400	-
Food Factor 2		0 ± 0.8	-0.1 ± 0.87	0.09 ± 0.73	0.344	-
zBMI		-0.24 ± 1.09	-0.18 ± 1.01	-0.29 ± 1.17	0.543	-
Child	Internalizing	3.49 ± 2.9	4.41 ± 3.23	2.68 ± 2.31	0.001*	<0.001*
	Externalizing	5.27 ± 3	5.75 ± 2.92	4.85 ± 3.04	0.165	<0.001*
	Prosocial	8.42 ± 1.38	8.55 ± 1.38	8.3 ± 1.37	0.356	0.830
Mother	Internalizing	2.38 ± 2.55	2.56 ± 2.62	2.22 ± 2.49	0.520	-
	Externalizing	3.42 ± 2.88	2.94 ± 2.58	3.85 ± 3.08	0.165	-
	Prosocial	8.39 ± 1.52	8.91 ± 1.08	7.95 ± 1.71	0.002*	-
Categorical variable			No / Yes		Chi-square test Girl *vs* Boy	
Antibiotics		129 / 8	61 / 3	68 / 5	0.862	
Diarrhea		53 / 84	30 / 34	23 / 50	0.287	
Constipation		118 /19	53 / 11	65 / 8	0.632	

† refers to comparisons for the total N = 137 children.

* indicates FDR-adjusted $p < 0.1$.

Child gender-related differences in gut microbiota composition

A total of 186 genus-level microbial taxa were observed. Alpha and beta diversity, as well as abundances of individual taxa, were compared between boys and girls. No significant gender differences were observed in alpha diversity as measured by Chao1, Shannon, and phylogenetic diversity indices (Figure S2). Regarding beta diversity, significant

compositional differences between genders were observed using weighted UniFrac distance and Bray-Curtis dissimilarity based on relative abundance data, and using the Aitchison distance based on absolute abundance data (Figure S3 and Figure S4, respectively). Gender and zBMI as well as mother-reported internalizing behavior jointly and significantly explained 3.6% of cumulative variance in the gut microbiota, when using the Bray-Curtis dissimilarity based on relative abundance data (Figure S5a and b). As for Bray-Curtis dissimilarity based absolute abundance data, only zBMI significantly explained 1.9% of variance (Figure S5c). No genus-level microbial taxa were differentially abundant after correcting for multiple tests.

Associations between the gut microbiota and internalizing, externalizing, and prosocial behavioral measures: without child gender-related differences in associations

Alpha diversity and behavior

The associations between alpha diversity and behavioral measures were assessed by Bayesian linear regression models (behavioral measures in relation to additional variables are displayed in Figure S6). Chao1 and Shannon diversity showed negative relations to child-reported prosocial behavior (Figure 2), indicating that higher alpha diversity was linked to less prosocial behavior at the age of 12 years. However, it should be noted that the significance of the relations differed between models.

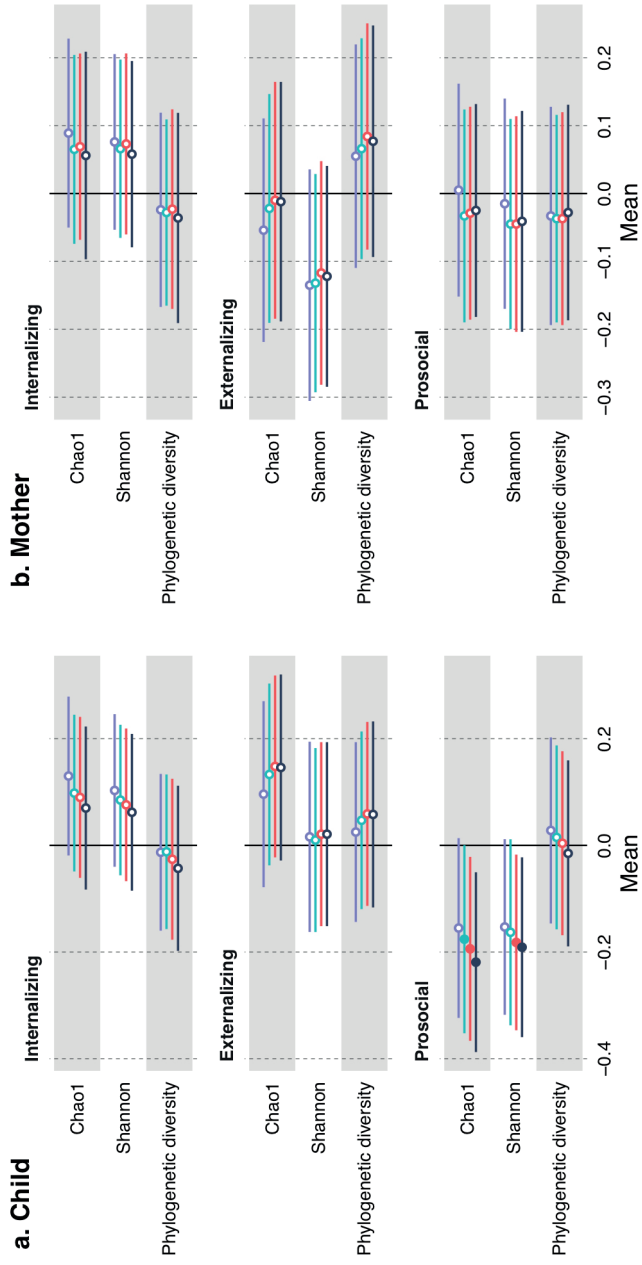

Figure 2. The relations between alpha diversity and behavioral measures. Relations were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: purple, Model 0; lake blue, Model 1; Red, Model 2; Black, Model 3), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations.

Relative abundances and behavior

The associations were evaluated for a total of 84 genus-level microbial taxa, which were prevalent in more than 10% of participants. Overall, 1.2% to 13.1% of microbiota-behavior associations were significant when using relative abundances (Table 2). In both child and maternal reports, more associations were significant in internalizing behavior (4.8% to 13.1%) than other behavioral measures (1.2% to 4.8%). The numbers of significant relations varied between models and reports, indicating that different additional variables and reporters influenced the significances of microbial predictors. As displayed in Table 3, 57.1% to 72.6% of associations were in the same direction using child and maternal reports, and few same-directional relations were significant.

Table 2. Number of significant estimates as measured by Bayesian models.

	Significant associations N					
	Child			Mother		
	Internalizing	Externalizing	Prosocial	Internalizing	Externalizing	Prosocial
Significant associations between genus-level microbial taxa and behavior (without the interaction of gender)						
Relative abundance						
Model 0	8 (9.5%)	1 (1.2%)	3 (3.6%)	11 (13.1%)	3 (3.6%)	3 (3.6%)
Model 1	4 (4.8%)	1 (1.2%)	3 (3.6%)	7 (8.3%)	3 (3.6%)	1 (1.2%)
Model 2	6 (7.1%)	2 (2.4%)	3 (3.6%)	9 (10.7%)	3 (3.6%)	2 (2.4%)
Model 3	6 (7.1%)	2 (2.4%)	4 (4.8%)	8 (9.5%)	1 (1.2%)	1 (1.2%)
Absolute abundance						
Model 0	5 (6%)	3 (3.6%)	5 (6%)	3 (3.6%)	4 (4.8%)	3 (3.6%)
Model 1	5 (6%)	5 (6%)	7 (8.3%)	3 (3.6%)	7 (8.3%)	2 (2.4%)
Model 2	4 (4.8%)	4 (4.8%)	7 (8.3%)	3 (3.6%)	6 (7.1%)	3 (3.6%)
Model 3	5 (6%)	5 (6%)	7 (8.3%)	3 (3.6%)	4 (4.8%)	3 (3.6%)
Significant gender- related differences in associations between taxa and behavior (with the interaction of gender)						
Relative abundance						
Model 1	4 (4.8%)	5 (6%)	10 (11.9%)	2 (2.4%)	11 (13.1%)	6 (7.1%)
Model 2	4 (4.8%)	6 (7.1%)	11 (13.1%)	2 (2.4%)	10 (11.9%)	5 (6%)
Model 3	3 (3.6%)	6 (7.1%)	8 (9.5%)	2 (2.4%)	10 (11.9%)	7 (8.3%)
Absolute abundance						
Model 1	8 (9.5%)	2 (2.4%)	6 (7.1%)	6 (7.1%)	4 (4.8%)	10 (11.9%)
Model 2	7 (8.3%)	1 (1.2%)	8 (9.5%)	5 (6%)	5 (6%)	9 (10.7%)
Model 3	8 (9.5%)	2 (2.4%)	7 (8.3%)	3 (3.6%)	5 (6%)	9 (10.7%)

The Bayesian estimates were assessed in N = 84 genus-level microbial taxa prevalent in more than 10% of all participants. Proportions in brackets are the ratios of the number to 84.

Table 3. Similarity of Bayesian estimates between child and maternal reports.

	Child *vs* Mother					
	Same direction N*			Same direction and significant N†		
	Internalizing	Externalizing	Prosocial	Internalizing	Externalizing	Prosocial
Associations between genus-level microbial taxa and behavior (without the interaction of gender)						
Relative abundance						
Model 0	51 (60.7%)	60 (71.4%)	49 (58.3%)	3 (3.6%)	1 (1.2%)	0 (0%)
Model 1	51 (60.7%)	61 (72.6%)	48 (57.1%)	0 (0%)	0 (0%)	0 (0%)
Model 2	52 (61.9%)	60 (71.4%)	49 (58.3%)	1 (1.2%)	0 (0%)	0 (0%)
Model 3	53 (63.1%)	60 (71.4%)	50 (59.5%)	0 (0%)	0 (0%)	0 (0%)
Absolute abundance						
Model 0	57 (67.9%)	61 (72.6%)	55 (65.5%)	0 (0%)	1 (1.2%)	1 (1.2%)
Model 1	57 (67.9%)	58 (69%)	50 (59.5%)	0 (0%)	3 (3.6%)	1 (1.2%)
Model 2	58 (69%)	57 (67.9%)	50 (59.5%)	0 (0%)	2 (2.4%)	1 (1.2%)
Model 3	56 (66.7%)	57 (67.9%)	50 (59.5%)	0 (0%)	3 (3.6%)	2 (2.4%)
Gender-related differences in associations between taxa and behavior (with the interaction of gender)						
Relative abundance						
Model 1	60 (71.4%)	67 (79.8%)	46 (54.8%)	0 (0%)	2 (2.4%)	2 (2.4%)
Model 2	58 (69%)	66 (78.6%)	46 (54.8%)	0 (0%)	3 (3.6%)	1 (1.2%)
Model 3	69 (82.1%)	67 (79.8%)	45 (53.6%)	0 (0%)	3 (3.6%)	1 (1.2%)
Absolute abundance						
Model 1	62 (73.8%)	61 (72.6%)	49 (58.3%)	1 (1.2%)	0 (0%)	1 (1.2%)
Model 2	62 (73.8%)	62 (73.8%)	52 (61.9%)	0 (0%)	0 (0%)	1 (1.2%)
Model 3	61 (72.6%)	61 (72.6%)	49 (58.3%)	2 (2.4%)	1 (1.2%)	1 (1.2%)

The Bayesian estimates were assessed in N = 84 genus-level microbial taxa prevalent in more than 10% of all participants. Proportions in brackets are the ratios of the number to 84.
* indicates that the mean values of the estimates are in the same direction for child and maternal reports.
† refers to significant estimates that are in the same direction for child and maternal reports.

Relations between microbial taxa and behavior that were significant for at least one behavioral scale are displayed in Figure 3. Below, we present all taxa that were significantly associated with a behavioral measure as reported by either child or mother:

Internalizing behavior

Christensenellaceae R-7 group, *Ruminococcus torques* group, *Romboutsia*, and *Turicibacter* were positively related to internalizing behavior in child reports. Microbial taxa, including *Eubacterium xylanophilum* group, genus CAG:56 within *Lachnospiraceae* family, *Roseburia*, *Ruminococcaceae* UCG-004, *Ruminococcus* 1, and *Parasutterella*, were positively associated with mother-reported internalizing behavior.

Externalizing behavior

Across all tested models, *Barnesiella* was negatively associated with child-reported externalizing behavior, and similarly, a reverse relation was noted between *Odoribacter* and externalizing behavior in maternal reports. Additionally, we found *Prevotella* 9 positively associated with mother-reported externalizing behavior in all tested conditions except for Model 3 (sensitivity analysis).

Prosocial behavior

Blautia and *Parasutterella* were negatively linked to child-reported prosocial behavior, whereas *Lachnospiraceae* ND3007 group exhibited positive relations to maternal reports of prosocial behavior.

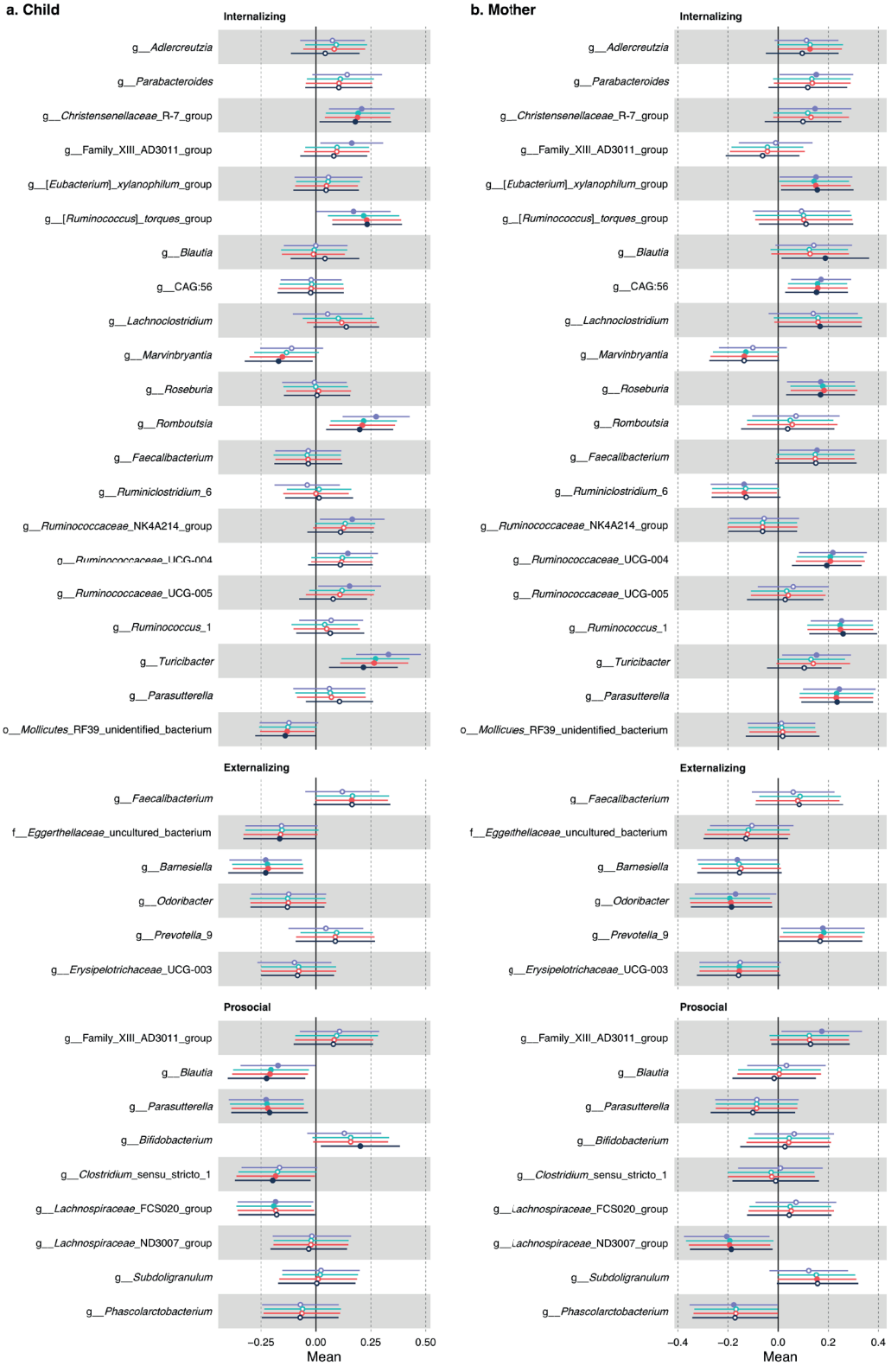
a. Child
b. Mother
Internalizing
g__Adlercreutzia
g__Parabacteroides
g__Christensenellaceae_R-7_group
g__Family_XIII_AD3011_group
g__[Eubacterium]_xylanophilum_group
g__[Ruminococcus]_torques_group
g__Blautia
g__CAG:56
g__Lachnoclostridium
g__Marvinbryantia
g__Roseburia
g__Romboutsia
g__Faecalibacterium
g__Ruminiclostridium_6
g__Ruminococcaceae_NK4A214_group
g__Ruminococcaceae_UCG-004
g__Ruminococcaceae_UCG-005
g__Ruminococcus_1
g__Turicibacter
g__Parasutterella
o__Mollicutes_RF39_unidentified_bacterium
Externalizing
g__Faecalibacterium
f__Eggerthellaceae_uncultured_bacterium
g__Barnesiella
g__Odoribacter
g__Prevotella_9
g__Erysipelotrichaceae_UCG-003
Prosocial
g__Family_XIII_AD3011_group
g__Blautia
g__Parasutterella
g__Bifidobacterium
g__Clostridium_sensu_stricto_1
g__Lachnospiraceae_FCS020_group
g__Lachnospiraceae_ND3007_group
g__Subdoligranulum
g__Phascolarctobacterium
−0.25
0.00
0.25
0.50
−0.4
−0.2
0.0
0.2
0.4
Mean

Figure 3. The relations between genus-level microbial taxon relative abundances and behavioral measures. Relations were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: purple, Model 0; lake blue, Model 1; Red, Model 2; Black, Model 3), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Only microbial taxa prevalent in more than 10% of all subjects and significant for at least one behavioral scale, are displayed in the figure.

Absolute abundances and behavior

Regarding absolute abundance-based outcomes, 2.4% to 8.3% of microbiota-behavior associations were significant (Table 2). More associations were significant in prosocial behavior (6% to 8.3%) than other behavioral measures (3.6% to 6%) as measured by child reports, while in maternal reports, more relations were significant in externalizing behavior (4.8% to 8.3%) compared to other measures (2.4% to 3.6%). Different numbers of significances were observed between models and reports, similar to the findings based on relative abundances. More than half of the tested microbial taxa showed the same direction in microbiota-behavior associations between child-mother reports (Table 3). Of these, several taxa, which were mainly related to externalizing behavior, showed same-directional associations that are significant.

Microbial taxa, with significant relations to at least one behavioral scale, are displayed in Figure 4. Significant taxa in all models are summarized below:

Internalizing behavior

Fusicatenibacter and *Butyricicoccus* were inversely associated with internalizing behavior in child reports. A negative relation was also discerned between *Coprococcus* 3 and internalizing behavior reported by mother. Additionally, mother-reported internalizing behavior was positively linked to *Ruminococcaceae* UCG-004 and *Parasutterella*.

Externalizing behavior

Ruminococcaceae UCG-013 and *Tyzzerella* 3 were reversely related to child-reported externalizing behavior. In contrast, *Lachnospiraceae* FSC020 group showed positive relations. Regarding maternal reports, negative associations were observed between *Odoribacter* and externalizing behavior, while positive relations were discerned in *Lachnospiraceae* FSC020 group, conforming to the findings in child reports.

Prosocial behavior

Child-reported prosocial behavior was negatively linked to the absolute abundances of four microbial taxa, including *Parasutterella*, *Alistipes*, *Parabacteroides*, and *Eubacterium xylanophilum* group, of which the last taxon also exhibited reverse relations to mother-reported prosocial behavior.

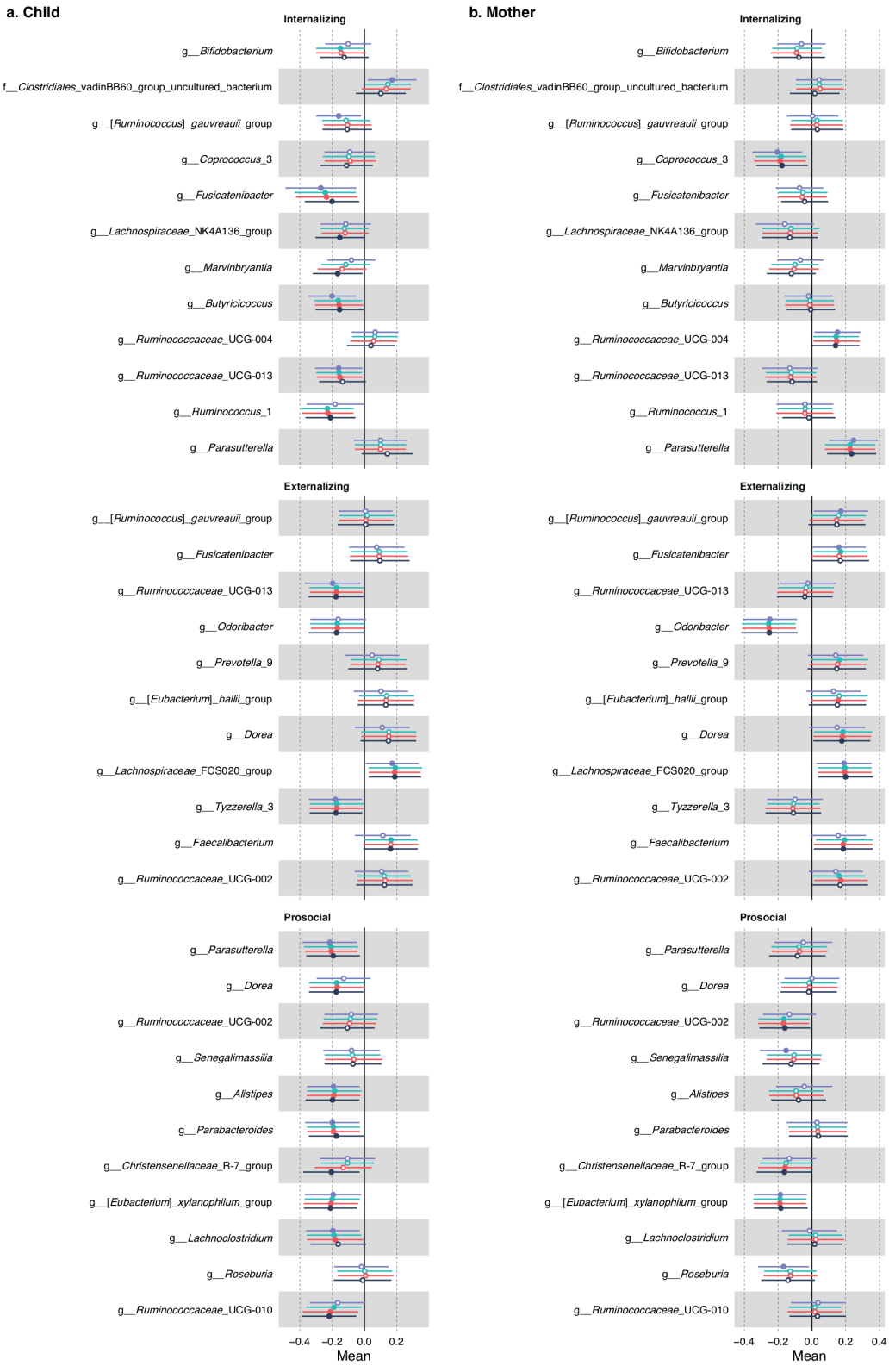
a. Child
b. Mother
Internalizing
g__Bifidobacterium
f__Clostridiales_vadinBB60_group_uncultured_bacterium
g__[Ruminococcus]_gauvreauii_group
g__Coprococcus_3
g__Fusicatenibacter
g__Lachnospiraceae_NK4A136_group
g__Marvinbryantia
g__Butyricicoccus
g__Ruminococcaceae_UCG-004
g__Ruminococcaceae_UCG-013
g__Ruminococcus_1
g__Parasutterella
Externalizing
g__[Ruminococcus]_gauvreauii_group
g__Fusicatenibacter
g__Ruminococcaceae_UCG-013
g__Odoribacter
g__Prevotella_9
g__[Eubacterium]_hallii_group
g__Dorea
g__Lachnospiraceae_FCS020_group
g__Tyzzerella_3
g__Faecalibacterium
g__Ruminococcaceae_UCG-002
Prosocial
g__Parasutterella
g__Dorea
g__Ruminococcaceae_UCG-002
g__Senegalimassilia
g__Alistipes
g__Parabacteroides
g__Christensenellaceae_R-7_group
g__[Eubacterium]_xylanophilum_group
g__Lachnoclostridium
g__Roseburia
g__Ruminococcaceae_UCG-010
−0.4 −0.2 0.0 0.2
0.4
Mean

Figure 4. The relations between genus-level microbial taxon absolute abundances and behavioral measures. Relations were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: purple, Model 0; lake blue, Model 1; Red, Model 2; Black, Model 3), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Only microbial taxa prevalent in more than 10% of all subjects and significant for at least one behavioral scale, are displayed in the figure.

Differences between relative and absolute abundances

The numbers of significant microbiota-behavior associations differed between relative and absolute abundances (Table 2). A higher number of significant associations in child-mother reported internalizing behavior were observed with relative abundances (4.8% to 13.1%) than with absolute abundances (3.6% to 6%). Contrarily, absolute abundances predicted more significances in externalizing and prosocial behavior in both reports. More than half of the microbial taxa (ranging from 52.4% to 76.2%) displayed the same direction in microbiota-behavior relations between abundance measures (Table 4). However, few of these same-directional relations were significant for both abundance data.

Table 4. Similarity of Bayesian estimates between relative and absolute abundances.

	Relative *vs* Absolute abundances					
	Same direction N*			Same direction and significant N†		
	Internalizing	Externalizing	Prosocial	Internalizing	Externalizing	Prosocial
Associations between genus-level microbial taxa and behavior (without the interaction of gender)						
Child						
Model 0	64 (76.2%)	56 (66.7%)	60 (71.4%)	0 (0%)	0 (0%)	1 (1.2%)
Model 1	56 (66.7%)	59 (70.2%)	55 (65.5%)	0 (0%)	0 (0%)	1 (1.2%)
Model 2	56 (66.7%)	60 (71.4%)	55 (65.5%)	0 (0%)	0 (0%)	1 (1.2%)
Model 3	50 (59.5%)	59 (70.2%)	61 (72.6%)	1 (1.2%)	0 (0%)	1 (1.2%)
Mother						
Model 0	52 (61.9%)	48 (57.1%)	55 (65.5%)	2 (2.4%)	1 (1.2%)	0 (0%)
Model 1	57 (67.9%)	44 (52.4%)	63 (75%)	2 (2.4%)	2 (2.4%)	0 (0%)
Model 2	55 (65.5%)	45 (53.6%)	62 (73.8%)	2 (2.4%)	1 (1.2%)	0 (0%)
Model 3	57 (67.9%)	44 (52.4%)	63 (75%)	2 (2.4%)	1 (1.2%)	0 (0%)

Gender-related differences in associations between taxa and behavior (with the interaction of gender)						
Child						
Model 1	61 (72.6%)	64 (76.2%)	63 (75%)	1 (1.2%)	1 (1.2%)	2 (2.4%)
Model 2	58 (69%)	66 (78.6%)	66 (78.6%)	1 (1.2%)	1 (1.2%)	4 (4.8%)
Model 3	59 (70.2%)	65 (77.4%)	63 (75%)	1 (1.2%)	1 (1.2%)	1 (1.2%)
Mother						
Model 1	65 (77.4%)	70 (83.3%)	59 (70.2%)	1 (1.2%)	0 (0%)	3 (3.6%)
Model 2	65 (77.4%)	71 (84.5%)	60 (71.4%)	1 (1.2%)	0 (0%)	2 (2.4%)
Model 3	64 (76.2%)	72 (85.7%)	59 (70.2%)	0 (0%)	0 (0%)	3 (3.6%)

The Bayesian estimates were assessed in N = 84 genus-level microbial taxa prevalent in more than 10% of all participants. Proportions in brackets are the ratios of the number to 84.
* indicates that the mean values of the estimates are in the same direction for relative and absolute abundances.
† refers to significant estimates that are in the same direction for relative and absolute abundances.

Microbiota-derived metabolites and behavior

In addition to gut microbiota composition, we also assessed behavioral links to microbiota-derived fecal metabolites, including lactate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, total SCFAs (including acetate, propionate, and butyrate), total BCFAs (including isobutyrate and isovalerate), and the ratio of total BCFAs to total SCFAs (concentrations of the metabolites and correlations between the metabolites are displayed in Table S3 and Figure S7, respectively). No significant links were observed, except the negative relations between child-reported externalizing behavior and the ratio of total BCFAs to total SCFAs (but insignificant for the independent relation as measured by Model 0) (Figure 5).

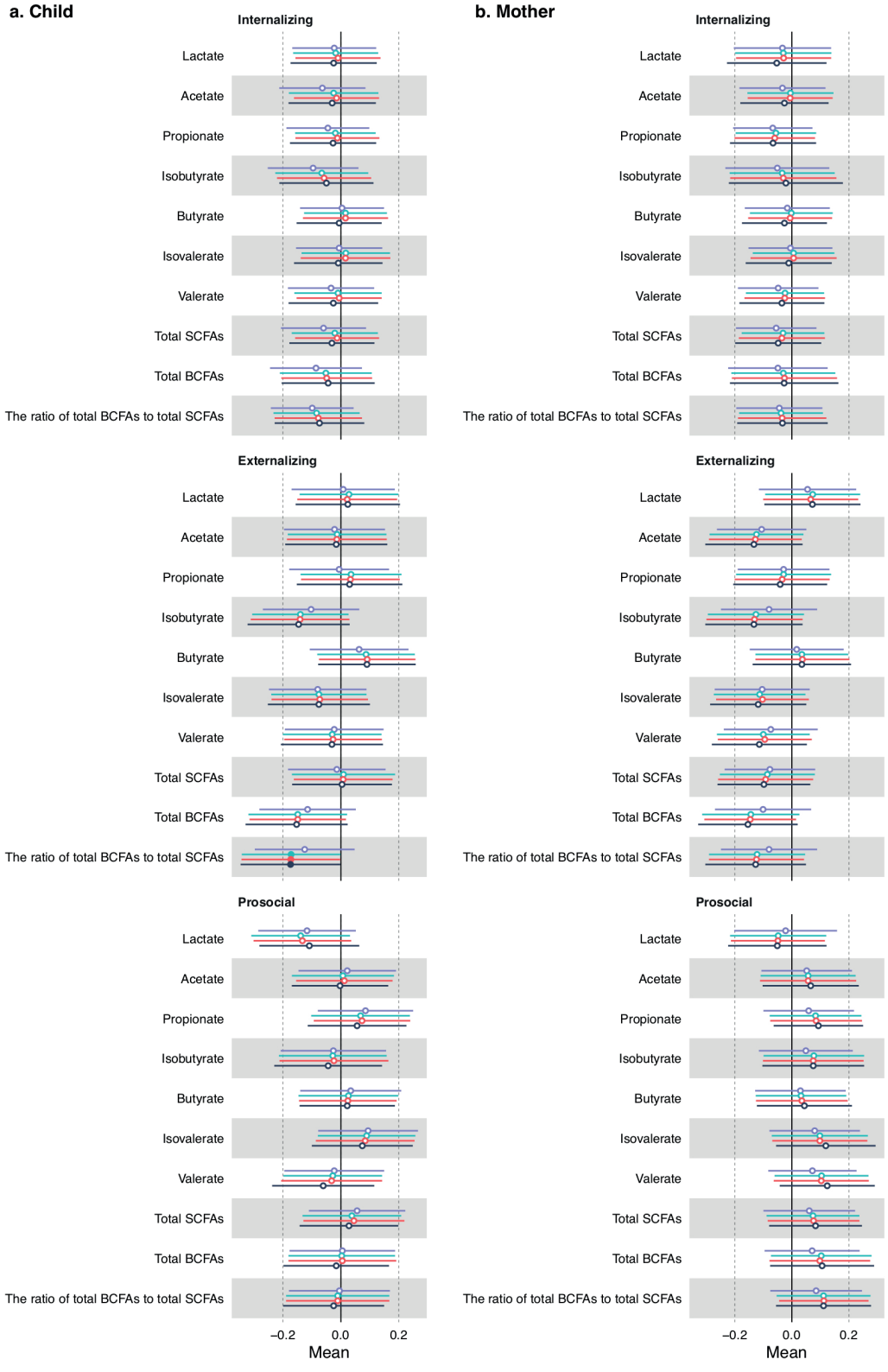
a. Child
b. Mother
Internalizing
Externalizing
Prosocial
Lactate
Acetate
Propionate
Isobutyrate
Butyrate
Isovalerate
Valerate
Total SCFAs
Total BCFAs
The ratio of total BCFAs to total SCFAs
−0.2
0.0
0.2
Mean

Figure 5. The relations between microbiota-derived fecal metabolites and behavioral measures. Relations were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: purple, Model 0; lake blue, Model 1; Red, Model 2; Black, Model 3), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations.

Associations between the gut microbiota and internalizing, externalizing, and prosocial behavioral measures: gender-related differences in associations

To test whether child gender-related differences exist in behavioral relations to the microbial predictors, we performed similar Bayesian models with an extra interaction term consisting of gender (dummy-scored as girl = 0 and boy = 1) and the predictor on the same community samples.

Alpha diversity and behavior

No gender-biased relations were found between alpha diversity and behavioral measures (Figure S8).

Relative abundances and behavior

Child gender-related differences in the associations were measured on a total of 84 genus-level microbial taxa present in more than 10% of all participants. Depending on the behavior and the model, 3.6% to 13.1% of child-reported outcomes differed significantly between boys and girls, and 2.4% to 13.1% of mother-reported relations varied significantly between genders (Table 2). Furthermore, 53.6% to 82.1% of the associations were in the same direction for child and maternal reports, but few of these same-directional relations were significant for both reports (Table 3).

Microbial taxa showing significant gender-related differences in associations with behavioral measures at least once, are displayed in Figure S9. The taxa maintaining significance in all tested models are presented below:

Internalizing behavior

In child reports, high relative abundances of *Ruminococcus gauvreauii* group and *Ruminoclostridium* 5 were related to more internalizing behavior, while more *Parasutterella* was associated with fewer internalizing difficulties, in boys compared to girls. In maternal reports, more *Lachnospiraceae* UCG-004 was associated with more internalizing problems, while high levels of *Lachnospiraceae* FCS020 group were related to less internalizing behavior, in boys compared to girls.

Externalizing behavior

Gender biases were observed in the relations between a total of five microbial taxa and child-reported externalizing behavior: in boys, high relative abundances of *Ruminococcus gauvreauii* group and *Sutterella* were linked to more externalizing problems, while high levels of *Christensenellaceae* R-7 group and *Coprococcus* 1 and 3 were related to fewer externalizing difficulties. In maternal reports, links belonging to a total of nine taxa

varied between both genders: more taxa within *Eubacterium xylanophilum* group, *Lachnospiraceae* NK4A136 group, and *Sutterella* were associated with more externalizing problems in boys compared to girls; more *Alistipes*, *Streptococcus*, *Coprococcus* 3, *Ruminoclostridium* 9, *Ruminococcaceae* UCG-005, and *Dialister* were related to less externalizing behavior in boys compared to girls.

Prosocial behavior

We found seven microbial taxa in gender-specific relation to child-reported prosocial behavior: the increase of one group within the *Lachnospiraceae* family was associated with more prosocial behavior in boys compared to girls, while other taxa (i.e., *Coprococcus* 1, *Ruminoclostridium* 9, another group within *Lachnospiraceae* family, *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-002 and 003) showed reverse relations. In maternal reports, high levels of Family XIII AD3011 group and an unidentified bacterium within the *Enterobacteriaceae* family were linked to more prosocial behavior, while more *Ruminococcaceae* UCG-003 and UCG-013 were associated with lower prosociality, in boys compared to girls.

Absolute abundances and behavior

Overall, 1.2% to 9.5% of genus-level microbial taxa were observed with significant gender differences in relation to child-reported behavior (Table 2). Maternal reports showed that 3.6% to 11.9% of taxa significantly exhibited such gender biases. The same-directional gender differences were observed in 58.3% to 73.8% of the relations between child and maternal reports, although few taxa were significant in both reports.

In the following, we present taxa with significant gender-dependent relations in all tested models (Figure S10):

Internalizing behavior

In child reports, high absolute abundances of *Lachnospiraceae* ND3007 group and *Faecalibacterium* were related to more internalizing behavior, while more *Parasutterella* predicted less internalizing behavior, in boys compared to girls. More *Butyricicoccus* and *Ruminococcaceae* UCG-013 were associated with more mother-reported internalizing difficulties in boys compared to girls.

Externalizing behavior

High absolute abundances of *Coprococcus* 3 predicted less child-reported externalizing behavior in boys than girls. Similar gender-related differences were also discerned in associations between maternal reports and three microbial taxa, including Family XIII AD3011 group, an unidentified bacterium within the *Tenericutes* phylum, and an uncultured bacterium within *Mollicutes* order, with higher absolute abundances associated with less externalizing behavior in boys. Also, high levels of *Lachnospiraceae* UCG-004 were associated with more mother-reported externalizing problems in boys.

Prosocial behavior

In child reports, high absolute abundances of microbial taxa (i.e., *Fusicatenibacter*, *Lachnospiraceae* UCG-004, an uncultured bacterium within the *Mollicutes* order, *Paraprevotella*, and *Ruminococcaceae* UCG-003) were associated with lower prosociality in boys compared to girls. In maternal reports, several taxa (i.e., *Lachnospiraceae* ND3007,

Lachnospiraceae NK4A136 group, *Ruminococcus gauvreauii* group, *Lachnospira*, and *Ruminococcaceae* UCG-003) showed similar gender-related differences. Contrarily, high levels of an unidentified bacterium within the *Enterobacteriaceae* family, *Erysipelatoclostridium*, and *Desulfovibrio* were linked to more prosocial behavior in boys compared to girls.

Differences between relative and absolute abundances

The numbers of significant gender-related differences in microbiota-behavior associations varied between relative and absolute abundances (Table 2). Relative abundances showed more gender-specific differences in child-reported externalizing and prosocial behavior, as well as mother-reported externalizing behavior, whereas absolute abundances showed more differences in child-reported internalizing behavior, and mother-reported internalizing and prosocial behavior. Despite few gender-related differences being same-directional and significant between abundances, 69% to 85.7% of differences were in the same direction between abundance measures (Table 4).

Microbiota-derived fecal metabolites and behavior

Gender-related differences were not observed in the relations between metabolites and behavioral scales measured by child reports (Figure S11). Contrarily, gender differences were found for maternal reports, including a negative association between externalizing behavior and isovalerate, and positive associations between prosocial behavior and isovalerate and valerate.

Discussion

Our study aimed to examine child gender differences in gut microbiota composition in children at an early stage of puberty, and to explore the associations between their behavior and gut microbiota composition and microbiota-derived fecal metabolites, also contemplating potential gender-related differences in these associations. Regarding the first aim, our results did not reflect gender-specific differences in alpha diversity or genus-level microbial taxon abundances but showed modest gender-dependent variations in beta diversity at this age. As for the second aim, multiple relations were observed between child behavior and taxon relative and absolute abundances, while associations were almost absent between behavior and microbial diversity as well as microbiota-derived fecal metabolites.

Biological gender differences have been observed in adult gut microbiota composition in population-level studies (Ding & Schloss, 2014; Vandeputte et al., 2016), along with changes in abundances of specific microbial taxa, such as *Prevotella*, *Bifidobacterium*, *Akkermansia*, and *Ruminococcus* (Mueller et al., 2006; Oki et al., 2016; Takagi et al., 2019). This discrepancy is assumed to most probably be attributed to gonadal hormones (Valeri & Endres, 2021). However, relatively little is known on children at the onset of puberty, when multiple simultaneous changes are being initiated in physiology and behavior. Here too, differences in gonadal hormones were put forward as a hypothesis, leading to divergence in gut microbiota composition between boys and girls in early puberty (Valeri & Endres, 2021). In our study, we found slight gender-specific variance in beta diversity and no differences in alpha diversity or abundances of individual genus-level microbial taxa (both relative and

absolute abundances). In line with this, Falony et al. reported a significant but small amount of compositional variance (beta diversity) explained by biological gender, in two population-based adult cohorts (Falony et al., 2016). Importantly, our findings on alpha diversity align with those of a previous study in children at around age 11 (Yuan et al., 2020). Regarding relative abundances, Yuan et al. found 11 differentially abundant taxa (e.g., *Phascolarctobacterium*, *Parabacteroides*, and *Alistipes*, which were enriched in girls) between genders in a group of five- to 15-year-old Chinese children, by using the same statistical approach as we performed (i.e., Wilcoxon rank sum test, also called Mann Whitney U test) but without FDR corrections (Yuan et al., 2020). Furthermore, adult studies reflected apparently higher relative abundances of *Prevotellaceae* taxa in males, and higher levels of *Bifidobacterium*, *Akkermansia*, and *Ruminococcaceae* taxa in females (Oki et al., 2016; Takagi et al., 2019). Taken together, these results suggest that puberty might be the start for the gut microbiota to differentiate between genders. It awaits to be further explored if these subtle microbial differences in early puberty become stronger during later puberty and then stabilize when gonadal hormones reach a steady state in adulthood.

Our analyses on associations between microbiota and behavior showed no relations between alpha diversity and problem behavior, but negative links between alpha diversity and prosocial behavior in these pubertal children. In a study in infants, Carlson et al. showed increased alpha diversity at the age of one year to be associated with lower cognitive ability at the age of two years (Carlson et al., 2018). Notably, cognition, especially moral cognition, can evoke a boost of prosocial behavior (Eisenberg, Fabes, & Spinrad, 2006), and this might support our findings of a reverse relation between alpha diversity and prosociality. Other studies on infants and pre-schoolers found increased alpha diversity in relation to less internalizing behavior (Laue et al., 2021; Van De Wouw et al., 2022), a link that was not found in our study. However, this comparison between infants and preschoolers and pubertal children is not without risks, as in puberty both the gut microbiota and the brain are thought to experience a second period of rapid growth and change after the first years of life, potentially influencing some gut-brain interactions.

In correspondence with previous studies, our results also suggest multiple associations between microbial taxa and child behavior. More than half of these relations were observed to have the same direction between child and maternal reports or between relative and absolute abundances, despite only a minority of them being significant relations. Moreover, the different reporters and abundance types produced a various number of significant relations, depending on the behavioral scales. In the following, we discuss three microbial taxa (i.e., *Ruminococcaceae* UCG-004, *Parasutterella*, and *Odoribacter*) for which both relative and absolute abundances exhibited significant relations to at least one behavioral measure. Next, we specifically discuss the relations of *Prevotella* 9, as it has been put forward as a noteworthy microbial taxon in our earlier research (Ou et al., 2022).

In the present study, we found that higher relative and absolute abundances of *Ruminococcaceae* UCG-004 were related to more child internalizing behavior reported by mothers. Although rarely being reported in research about internalizing difficulties, increased relative abundances of *Ruminococcaceae* UCG-004 were found to be strongly

associated with more inattention symptoms in patients with attention-deficit/hyperactivity disorder (ADHD) at ages of 13 to 29 years (Szopinska-Tokov et al., 2020). An animal study also showed elevated anxiety-like behavior in germ-free mice receiving fecal microbiota transplantation (FMT) from ADHD donors (Tengeler et al., 2020). As cellulolytic bacteria (Flint, Bayer, Rincon, Lamed, & White, 2008), taxa within the *Ruminococcaceae* family are widely present in the human gut and several are known to be butyrate producers (Dalile et al., 2019). At the family level, depleted *Ruminococcaceae* was observed in patients with ASD (Liu et al., 2019) and bipolar disorder (Painold et al., 2019), and in mice with FMT from ADHD subjects (Tengeler et al., 2020). However, findings were not consistent between the taxa within this family (Tengeler et al., 2020), likely indicating divergent interplay mechanisms between *Ruminococcaceae* taxa.

In addition to *Ruminococcaceae* UCG-004, relative and absolute abundances of *Parasutterella* were positively related to mother-reported internalizing behavior. Moreover, both abundances of *Parasutterella* were negatively associated with child-reported prosocial behavior. Interestingly, overgrowing *Parasutterella* has been observed in patients with major depressive disorder (MDD) (Jiang et al., 2015). Furthermore, depressive symptoms might be at least partly attributed to higher proinflammatory cytokines stimulated by lipopolysaccharides from this Gram-negative bacterium (Amirkhanzadeh Barandouzi, Starkweather, Henderson, Gyamfi, & Cong, 2020). Recently, Yao et al. found that the interaction between *Parasutterella* abundances and dietary sugar consumption was modestly associated with less anxious symptoms and alleviated anxiety severity in adults (Yao et al., 2022). Additionally, *Parasutterella* could actively engage in the metabolism of bile acids (critical to digestion and absorption of fats) and tryptophan (an essential amino acid prevalent in dairy products and nuts, and a precursor of serotonin), further emphasizing the importance of dietary effects on child behavior (Ju, Kong, Stothard, & Willing, 2019).

Additionally, we found relative and absolute abundances of *Odoribacter* in reverse relations to mother-reported externalizing behavior. In accordance with these findings, *Odoribacter* depletions have been observed in children with ASD (Kang et al., 2018) and have been related to worse performance in elevated plus maze tests performed on mice with early adversity (Rincel et al., 2019). Notably, *Odoribacter* has the genetic potential for producing γ-aminobutyric acid (GABA; a primary inhibitory neurotransmitter) (Yunes et al., 2016). Reduced cerebral GABA concentrations have been found in children with ADHD (Edden, Crocetti, Zhu, Gilbert, & Mostofsky, 2012) and ASD (Rojas, Singel, Steinmetz, Hepburn, & Brown, 2014).

Apart from aforementioned genus-level microbial taxa, another noteworthy relation was found between higher relative abundances of *Prevotella* 9 and more mother-reported externalizing difficulties. This complies with our earlier findings in the same cohort that increased *Prevotella* 9 at ages six and ten years was associated with more externalizing problems at age ten (Ou et al., 2022). Even though the underlying mechanism remains unclear yet, an altered gut inflammatory status might be behind these associations (Fung, 2020). Recently, Iljazovic et al. found that one *Prevotella* species exacerbated gut inflammation in mice, characterized by reduced concentrations of SCFAs and raised levels

of pro-inflammatory cytokines (Iljazovic et al., 2021). However, it is important to be aware of the divergent roles that *Prevotella* spp. may play in various scenarios. Additionally, *Prevotella* was observed to be highly prevalent in non-westerners consuming plant-rich diets, and therefore supposed to exert beneficial effects on host health (Ley, 2016). Regarding child mental health, Loughman et al. observed a reduction in *Prevotella* in one-year-old infants related to more internalizing problems at age two (Loughman et al., 2020). Putting aside the specifics, previous and current results on relations between *Prevotella* and problem behavior point at this microbial taxon as an interesting target for future studies.

Regarding microbiota-derived fecal metabolites, we did not find any associations to the studied behavior, except for the reverse relations of the ratios (i.e., total BCFAs to total SCFAs) to child-reported externalizing behavior. Although this may appear striking, it is worth noting that despite fecal SCFAs being widely recognized as beneficial to general health, their roles in mental health have not been fully determined (Dalile et al., 2019; Mirzaei et al., 2021). For instance, increased propionate may partly underlie the pathology of ASD in some children (Mirzaei et al., 2021). In line with this, an *in vivo* study showed ASD-like symptoms in rats after intracerebroventricular injections of propionate (Shultz et al., 2009). Additionally, depression in adults was related to reduced levels of *Oscillibacter* species (Naseribafrouei et al., 2014), of which the main end product is valerate, structurally resembling GABA. However, follow-up assessments did not show decreased fecal valerate in depressed adults but increased levels of its isomer, isovalerate (Szczesniak et al., 2016). Until now, evidence is limited and less uniform in human research. In addition to being used as energy source by colonic epithelial cells, importantly, SCFAs can activate receptors on immune, enteroendocrine, and vagal nerve cells, indirectly impacting brain physiology and functions via a complex signaling network (Dalile et al., 2019). Finally, it is relevant to know that the production of some fecal metabolites, such as the SCFAs and BCFAs investigated in the present study, is largely influenced by the amount, category, and even structure of dietary ingredients (Deehan et al., 2020). This suggests that in future studies it will be essential to more comprehensively record metabolite levels and food consumption, such as by means of 24-hour recalls.

Regardless of the growing evidence linking the gut microbiota to child behavior, relatively few investigations have been conducted to examine gender differences in these relations (Aatsinki et al., 2019; Christian et al., 2015). In our study, different associations between the gut microbiota and behavioral outcomes were found in boys and girls, as explained by the significant interactions of child gender and the microbiota-related variables. In particular, we found three robust gender-related differences in microbiota-behavior associations, which were identical between relative and absolute abundances. These relations were between *Parasutterella* and child-reported internalizing behavior, *Coprococcus* 3 and child-reported externalizing behavior, and *Ruminococcaceae* UCG-003 and prosocial behavior reported by children and mothers. In addition to microbial abundances, we also observed fecal isovalerate levels in different relations to mother-reported externalizing and prosocial behavior. According to a study by Christian et al., surgency (an early temperamental trait that is predictive of subsequent externalizing

symptoms (Rothbart, Ahadi, & Hershey, 1994)) was positively related to alpha and beta diversity as well as *Ruminococcaceae* abundances in boys, and fear reactivity (another early temperamental trait negatively correlated with later externalizing problems (Rothbart et al., 1994)) was positively associated with *Rikenellaceae* abundances in girls before age three (Christian et al., 2015). Also Aatsinki et al. observed gender specificities between microbial taxa with varying compositional features in child temperament (Aatsinki et al., 2019). Additionally, higher relative abundances of *Actinobacteria* were observed in female MDD adults, while lower relative abundances of *Bacteroidetes* were found in male MDD adults (Chen et al., 2018). In sum, our findings and those of previous research accentuate the importance of considering child gender from early puberty onwards as an influential factor on associations between microbiota and behavior. The mechanisms underlying such relations need to be explored comprehensively in forthcoming research.

Our study should be considered with some strengths and limitations. The strengths include: (1) the use of preregistration; (2) the use of a relatively large group of low-risk early pubertal children; (3) the inclusion of both child and maternal reports; (4) the quantification of the concentrations of microbiota-derived fecal metabolites; (5) the utilization of multiple Bayesian linear regression models; (6) the focus on gender as a potential covariate to gut microbiota composition and a possible moderator of microbiota-behavior relations in children; and (7) the use of both relative and absolute abundances, emphasizing the necessity to take the total microbial load into account. Our limitations include: (1) due to financial constraints, only a restricted number of microbiota-derived fecal metabolites were analyzed. Future inclusion of other previously reported relevant metabolites, such as serotonin and GABA, or the use of non-targeted metabolomic approaches to profile all fecal metabolites, is recommended. Moreover, compared to feces, peripheral blood samples (unavailable in the current cohort) are thought to reflect biologically meaningful metabolite levels more straightforwardly; (2) primer bias may occur during qPCR (Jian et al., 2020); (3) the food frequency questionnaire provides fewer details of daily food consumption than other instruments; and (4) causal relationships cannot be determined in a cross-sectional observational study.

In conclusion, subtle and slight differences were observed in gut microbiota composition between boys and girls at an early stage of puberty. Whether these differences remain stable or become larger in adolescence remains to be determined. Gut microbiota composition was associated with problem and prosocial behavior in this low-risk community cohort, in gender-specific manners in some cases. Finally, this study validated our earlier findings in middle childhood that *Prevotella* 9 levels were positively associated with externalizing behavior. Considering the scarcity of investigations on the gut microbiota-behavior relations in low-risk pubertal populations, our study adds novel and relevant results that can serve as a basis for future research in this field.

Data availability statement

As the findings in this study are supported by datasets from an ongoing longitudinal cohort, these datasets currently cannot be made publicly available but are available upon request from C.deW. (Carolina.deWeerth@radboudumc.nl)

Acknowledgements

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Supplemental information

Imputation of missing values in zBMI

For a total of 43 participants who did not provide height and weight information at the studied age, we performed imputations following these equations: (1) when height and weight information was provided at both earlier and later ages (i.e., ten and 13 years, N=35), height and weight values for the current study were calculated based on $y_{height} = a * x_{Age} + b$ and $y_{weight} = a * x_{Age} + b$, where a is the slope and b is the intercept obtained from the linear equation of early and later ages, while x_{Age} indicates the age in days for the present study; (2) when height and weight values were recorded at either age ten years or age 13 years (N=8), the available data are directly used to calculate zBMI for the present study without imputation.

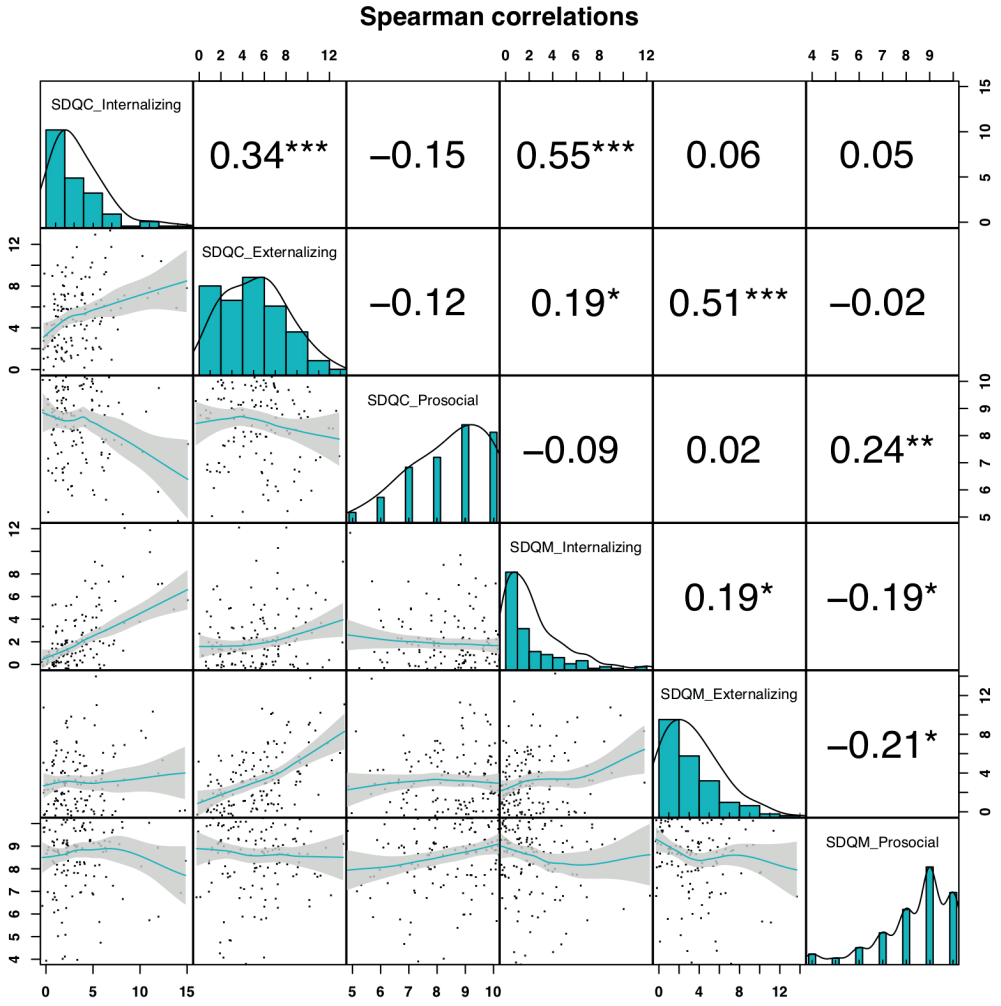

Figure S1. Spearman correlations between the SDQ scales. The distribution of each behavioral measure is displayed on the diagonal. Bivariate scatter plots of every two behavioral measures were shown on the bottom of the diagonal, with a fitted regression line in green. Correlation coefficients are shown on the top of the diagonal, plus their significances represented with asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Scales along x and y axes indicate behavioral scores. SDQ: the Strengths and Difficulties Questionnaire; C: child; M: maternal.

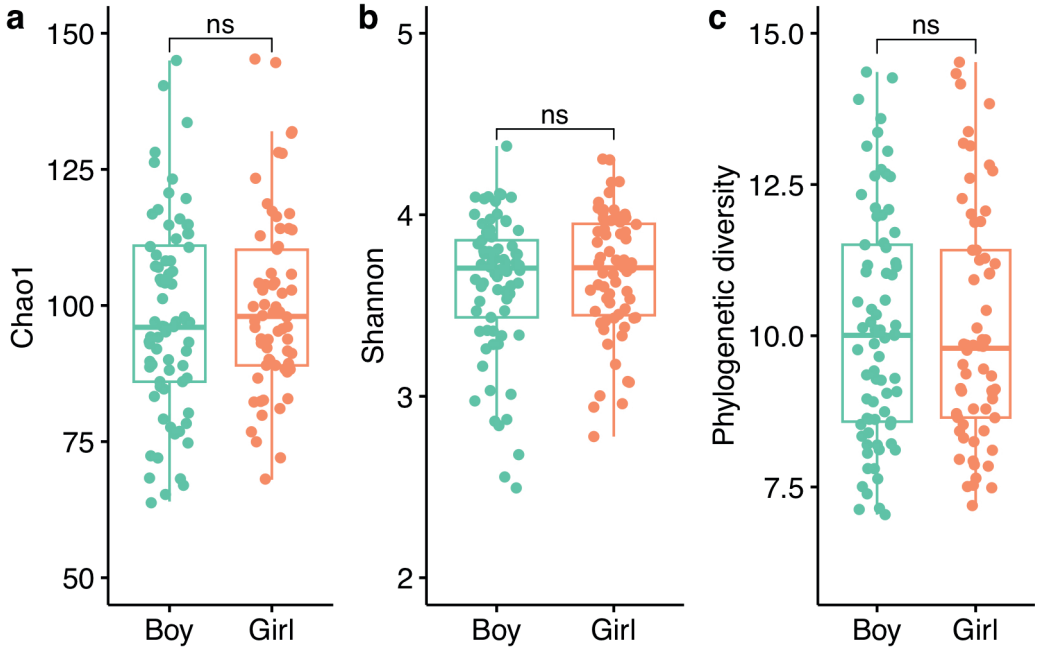

Figure S2. Alpha diversity in boys and girls, as measured by three different indices, including (a) Chao1, (b) Shannon, and (c) Phylogenetic diversity.

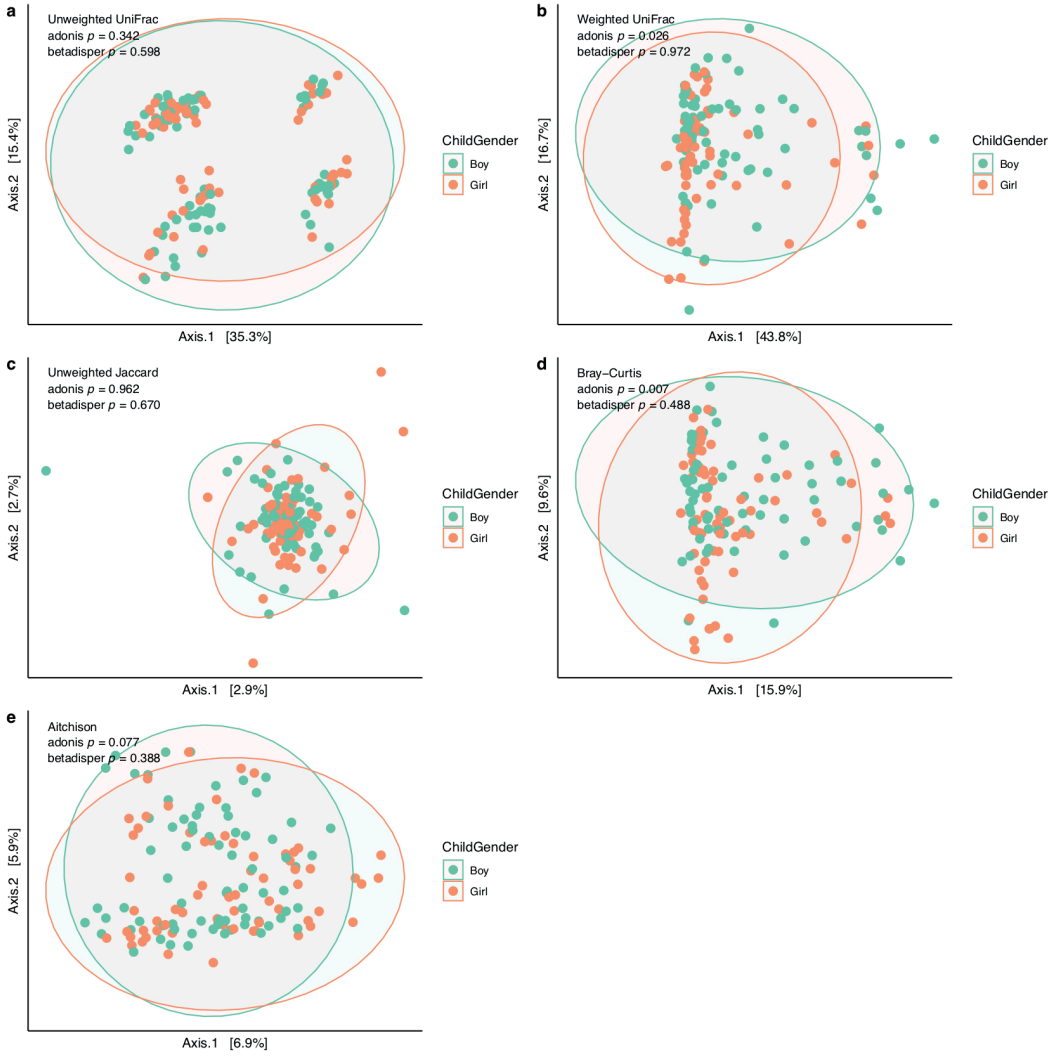

Figure S3. PCoA plots of relative abundance-based beta diversity at the genus level between genders. Adonis *p* values lower than 0.05 indicate significant differences in beta diversity between boys and girls. Betadisper *p* values lower than 0.05 suggest heterogeneous dispersions between boys and girls. Notes for the Aitchison distance, original ASV count data were clr-transformed and then calculated with the Euclidean distance.

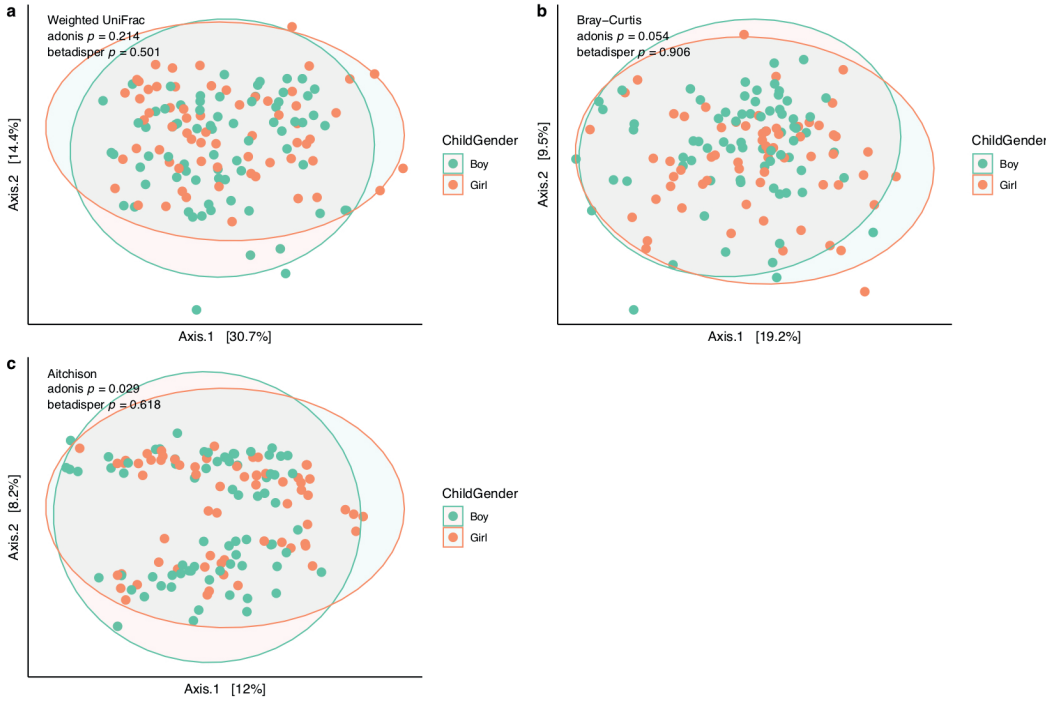

Figure S4. PCoA plots of absolute abundance-based beta diversity at the genus level between genders. Adonis *p* values lower than 0.05 indicate significant differences in beta diversity between boys and girls. Betadisper *p* values lower than 0.05 suggest heterogeneous dispersions between boys and girls. Note that unweighted UniFrac and Jaccard distances only consider the absence and presence of taxa, therefore the plots are the same to the ones using relative abundance data and are not displayed here. Except for the Aitchison distance using count data, absolute abundances were log-transformed before being used in PCoA plots.

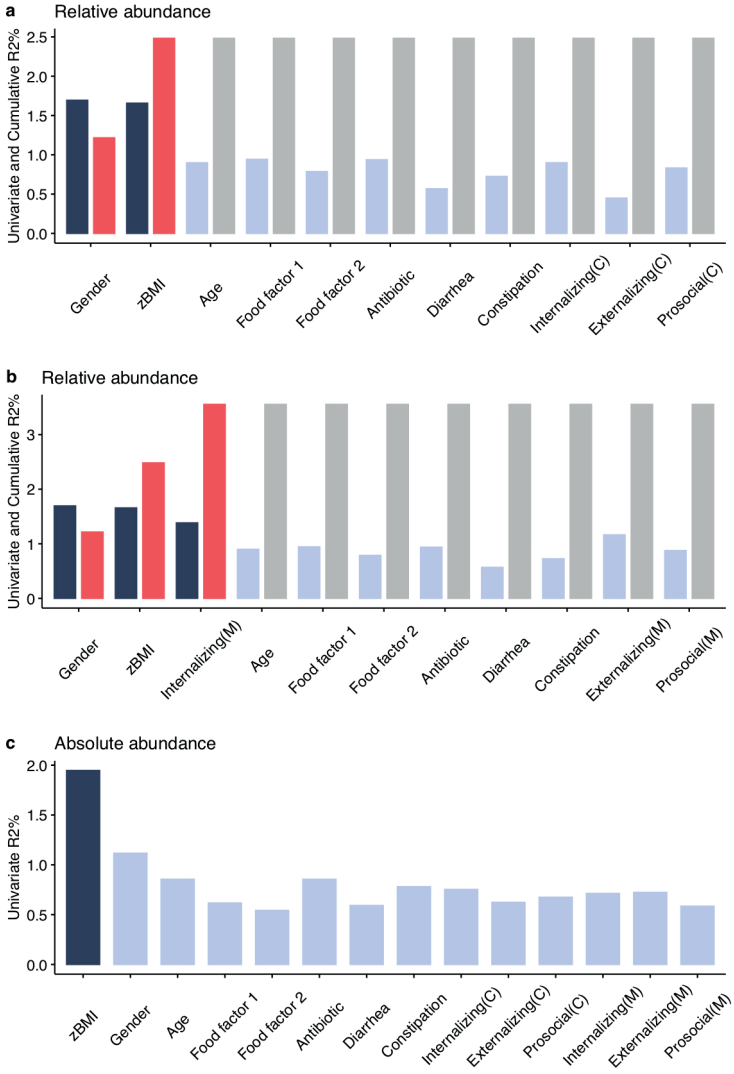

Figure S5. Univariate and cumulative variance (R^2%) in the gut microbiota explained by the additional variables and behavioral measures based on distance-based redundancy analysis. R^2% on the Bray-Curtis distance of relative abundance data, with child- and mother-reported variables displayed in (a) and (b), respectively. Left bars indicate univariate R^2% explained by an individual variable only. Black, $p < 0.05$; Purple, $p \geq 0.05$. Right bars refer to cumulative R^2% explained jointly by variables with $p < 0.05$, which were determined through the *ordiR2step* R function and colored in red. Variables with $p \geq 0.05$ were not included in the cumulative model and colored in gray. (c) R^2% on the Bray-Curtis distance of log-transformed absolute abundance data. Considering zBMI was the only variable exhibiting univariate R^2% with $p < 0.05$, we presented univariate R^2% of child- and mother-reported behavior in one graph and did not further calculate cumulative R^2%. C, child; M, mother.

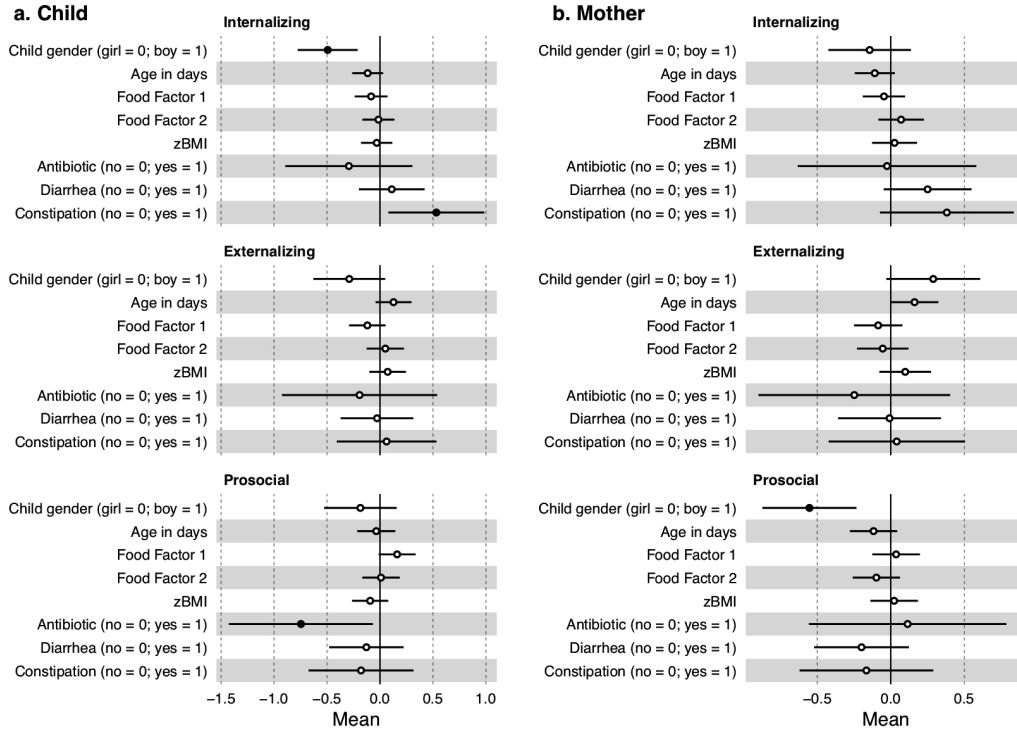

Figure S6. The independent relations between behavioral measures and child gender as well as additional variables. Relations were determined by Bayesian linear regression models, with lines and circles indicating 95% CIs and mean values of estimates. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that 95% CIs include 0, suggesting insignificant relations.

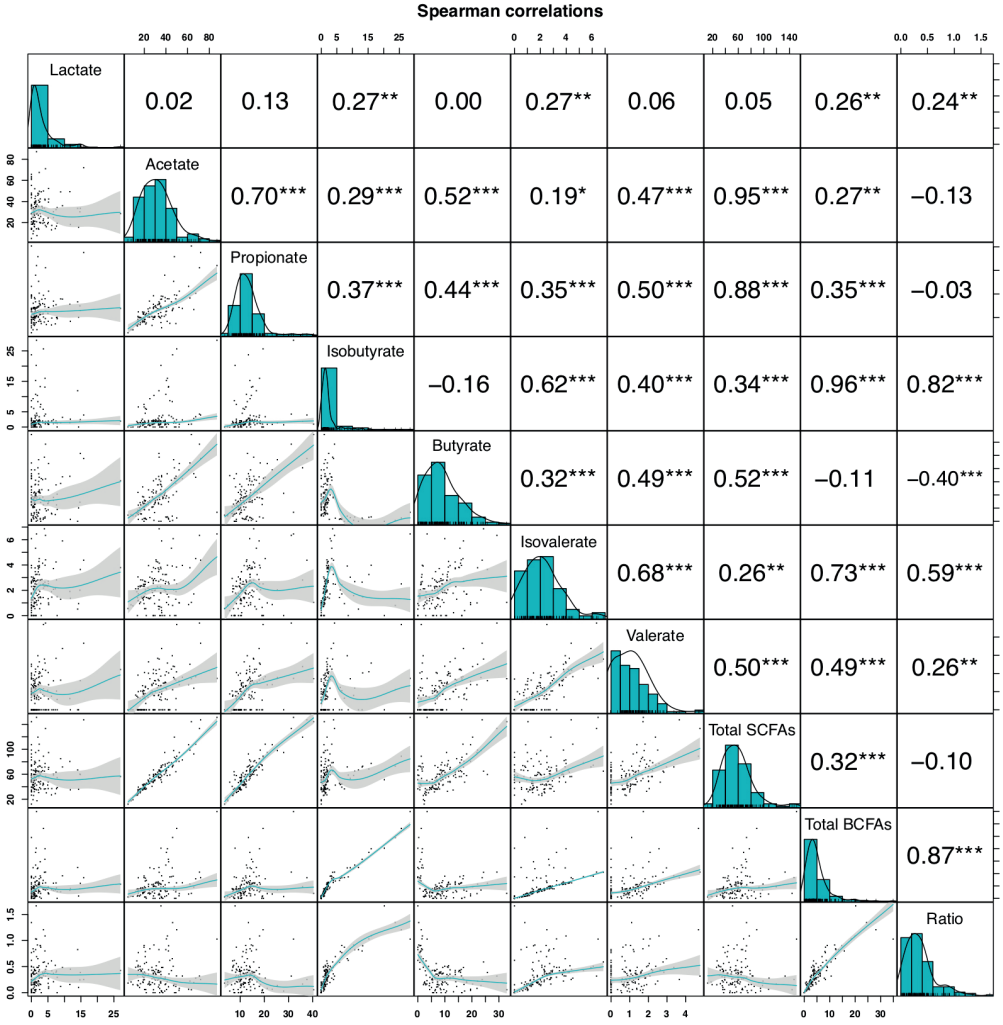

Figure S7. Spearman correlations between concentrations of microbiota-derived fecal metabolites across all subjects. Ratio indicates the ratio of total BCFAs (including isobutyrate and isovalerate) to total SCFAs (including acetate, propionate, and butyrate). The distribution of each metabolite is displayed on the diagonal. Bivariate scatter plots of every two metabolites are shown on the bottom of the diagonal, with a fitted regression line in green. Correlation coefficients are shown on the top of the diagonal, plus their significances represented with asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Scales along x and y axes indicate metabolite concentrations (or the ratio of total BCFAs to total SCFAs).

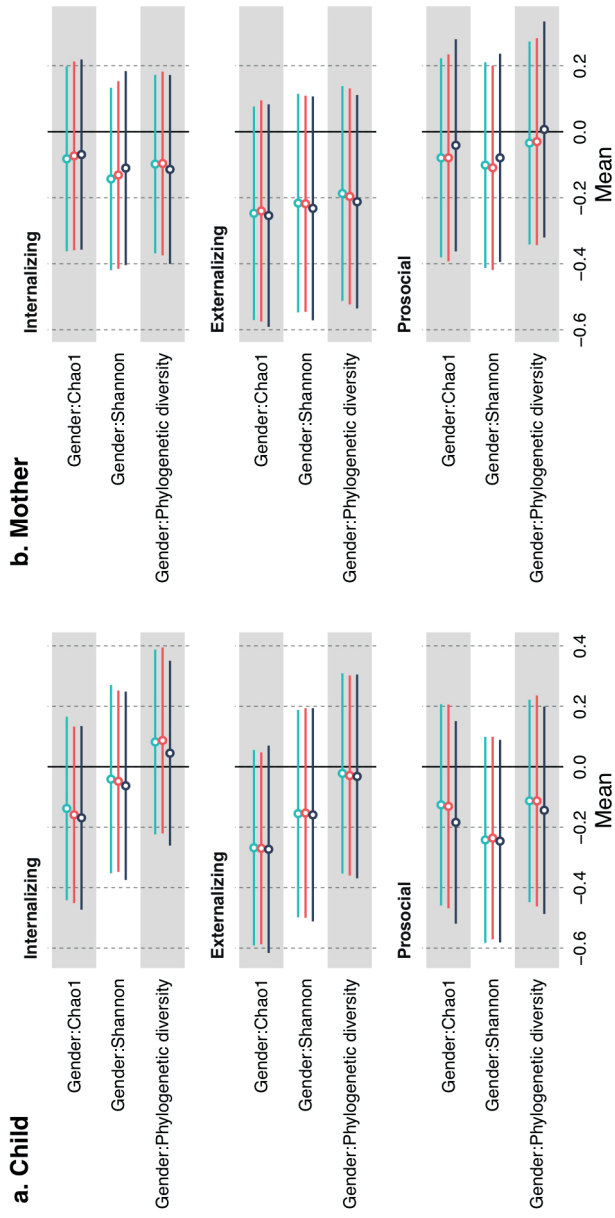

Figure S8. Gender-related differences in the relations between alpha diversity and the three behavioral measures. The interaction terms of child gender and alpha diversity were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: lake blue, Model 1 with the interaction; Red, Model 2 with the interaction; Black, Model 3 with the interaction), respectively. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Gender was dummy-scored as girl = 0 and boy = 1.

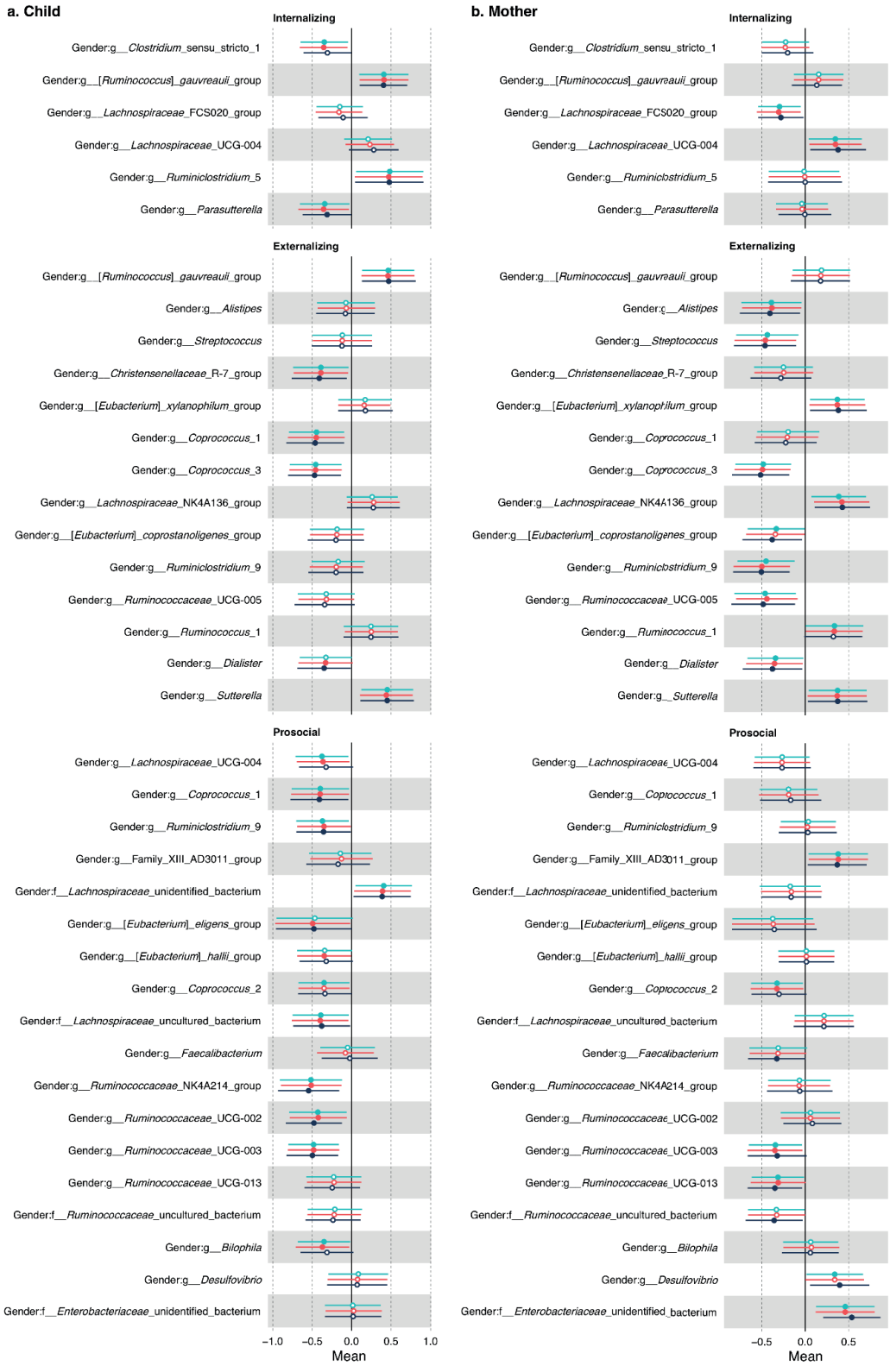
a. Child
b. Mother
Internalizing
Gender:g__Clostridium_sensu_stricto_1
Gender:g__[Ruminococcus]_gauvreauii_group
Gender:g__Lachnospiraceae_FCS020_group
Gender:g__Lachnospiraceae_UCG-004
Gender:g__Ruminiclostridium_5
Gender:g__Parasutterella
Externalizing
Gender:g__[Ruminococcus]_gauvreauii_group
Gender:g__Alistipes
Gender:g__Streptococcus
Gender:g__Christensenellaceae_R-7_group
Gender:g__[Eubacterium]_xylanophilum_group
Gender:g__Coprococcus_1
Gender:g__Coprococcus_3
Gender:g__Lachnospiraceae_NK4A136_group
Gender:g__[Eubacterium]_coprostanoligenes_group
Gender:g__Ruminiclostridium_9
Gender:g__Ruminococcaceae_UCG-005
Gender:g__Ruminococcus_1
Gender:g__Dialister
Gender:g__Sutterella
Prosocial
Gender:g__Lachnospiraceae_UCG-004
Gender:g__Coprococcus_1
Gender:g__Ruminiclostridium_9
Gender:g__Family_XIII_AD3011_group
Gender:f__Lachnospiraceae_unidentified_bacterium
Gender:g__[Eubacterium]_eligens_group
Gender:g__[Eubacterium]_hallii_group
Gender:g__Coprococcus_2
Gender:f__Lachnospiraceae_uncultured_bacterium
Gender:g__Faecalibacterium
Gender:g__Ruminococcaceae_NK4A214_group
Gender:g__Ruminococcaceae_UCG-002
Gender:g__Ruminococcaceae_UCG-003
Gender:g__Ruminococcaceae_UCG-013
Gender:f__Ruminococcaceae_uncultured_bacterium
Gender:g__Bilophila
Gender:g__Desulfovibrio
Gender:f__Enterobacteriaceae_unidentified_bacterium
−1.0
−0.5
0.0
0.5
1.0
Mean

Figure S9. Gender-related differences in the relations between genus-level microbial taxon relative abundances and the three behavioral measures. The interaction terms of child gender and relative abundances were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: lake blue, Model 1 with the interaction; Red, Model 2 with the interaction; Black, Model 3 with the interaction), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Only microbial taxa prevalent in more than 10% of all subjects and significant for at least one behavioral scale, are displayed in the figure. Gender was dummy-scored as girl = 0 and boy = 1. For instance, high relative abundances of *Ruminococcus gauvreauii* group predicted more child-reported internalizing behavior in boys than girls.


a. Child
b. Mother
Internalizing
Gender:g__Odoribacter
Gender:f__Muribaculaceae_uncultured_bacterium
Gender:g__Parabacteroides
Gender:g__Dorea
Gender:g__Fusicatenibacter
Gender:g__Lachnospiraceae_ND3007_group
Gender:g__Lachnospiraceae_NK4A136_group
Gender:g__Lachnospiraceae_UCG-004
Gender:f__Lachnospiraceae_uncultured_bacterium
Gender:g__[Eubacterium]_coprostanoligenes_group
Gender:g__Butyricicoccus
Gender:g__Faecalibacterium
Gender:g__Ruminococcaceae_UCG-013
Gender:g__Ruminococcus_1
Gender:g__Parasutterella
Externalizing
Gender:g__Lachnospiraceae_UCG-004
Gender:g__Butyricicoccus
Gender:g__Parasutterella
Gender:g__Family_XIII_AD3011_group
Gender:g__Coprococcus_3
Gender:f__Enterobacteriaceae_unidentified_bacterium
Gender:p__Tenericutes_unidentified_bacterium
Gender:o__Mollicutes_RF39_uncultured_bacterium
Prosocial
Gender:g__Fusicatenibacter
Gender:g__Lachnospiraceae_ND3007_group
Gender:g__Lachnospiraceae_NK4A136_group
Gender:g__Lachnospiraceae_UCG-004
Gender:g__Butyricicoccus
Gender:f__Enterobacteriaceae_unidentified_bacterium
Gender:o__Mollicutes_RF39_uncultured_bacterium
Gender:g__Paraprevotella
Gender:g__[Eubacterium]_eligens_group
Gender:g__[Eubacterium]_xylanophilum_group
Gender:g__[Ruminococcus]_gauvreauii_group
Gender:g__Lachnospira
Gender:g__Lachnospiraceae_UCG-001
Gender:g__Roseburia
Gender:g__Ruminococcaceae_UCG-003
Gender:g__Ruminococcaceae_UCG-004
Gender:g__Erysipelatoclostridium
Gender:g__Bilophila
Gender:g__Desulfovibrio
−0.5
0.0
0.5
Mean

Figure S10. Gender-related differences in the relations between genus-level microbial taxon absolute abundances and the three behavioral measures. The interaction terms of child gender and absolute abundances were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: lake blue, Model 1 with the interaction; Red, Model 2 with the interaction; Black, Model 3 with the interaction), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Only microbial taxa prevalent in more than 10% of all subjects and significant for at least one behavioral scale, are displayed in the figure. Gender was dummy-scored as girl = 0 and boy = 1. For instance, high absolute abundances of *Faecalibacterium* predicted more child-reported internalizing behavior in boys than girls.

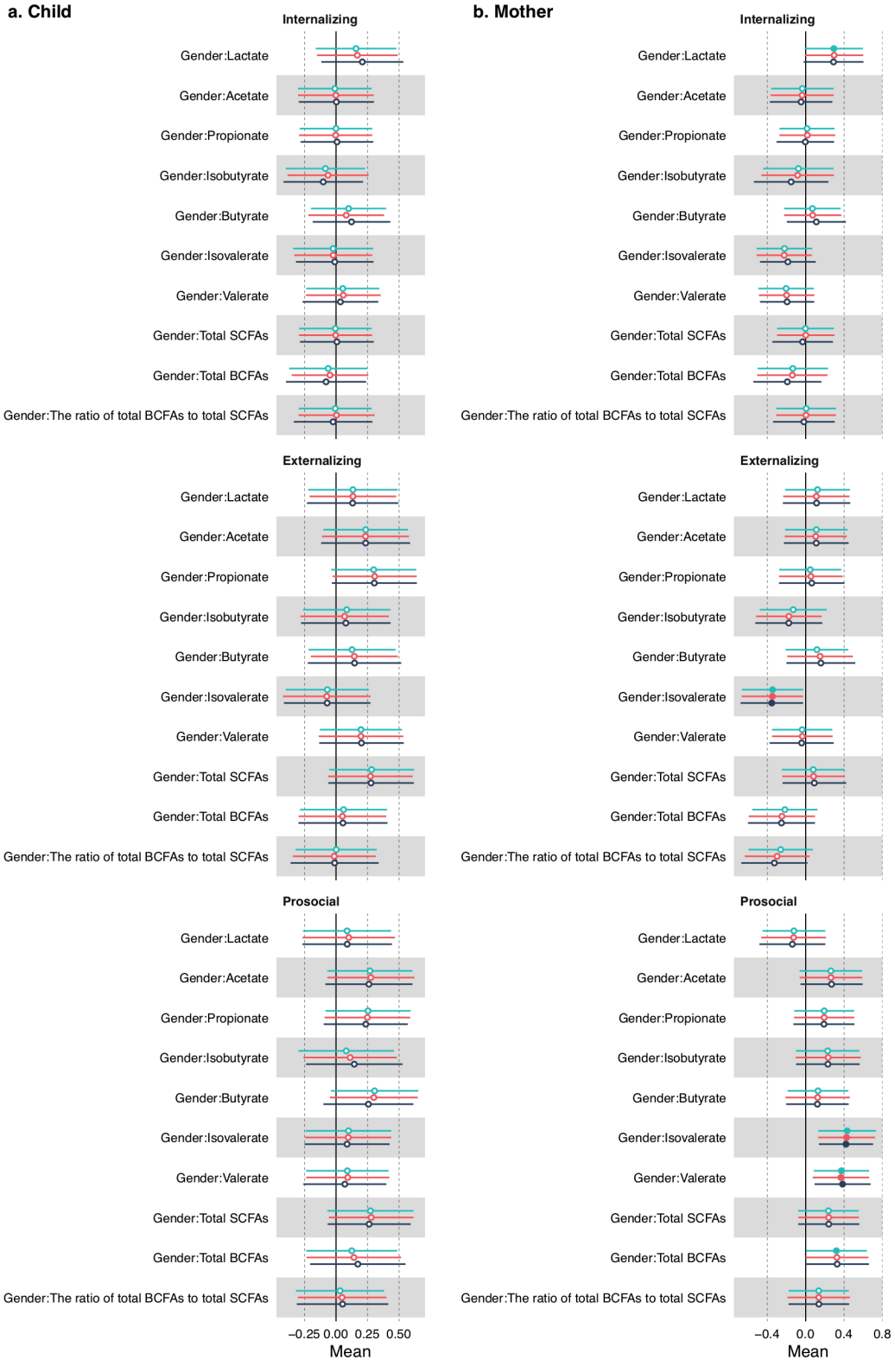
a. Child
b. Mother
Internalizing
Externalizing
Prosocial
Gender:Lactate
Gender:Acetate
Gender:Propionate
Gender:Isobutyrate
Gender:Butyrate
Gender:Isovalerate
Gender:Valerate
Gender:Total SCFAs
Gender:Total BCFAs
Gender:The ratio of total BCFAs to total SCFAs
−0.25 0.00 0.25 0.50
−0.4 0.0 0.4 0.8
Mean

Figure S11. Gender-related differences in the relations between microbiota-derived fecal metabolites and the three behavioral measures. The interaction terms of child gender and fecal metabolites were determined by Bayesian linear regression models, with differently colored lines and circles indicating 95% CIs and mean values of estimates (from top to bottom: lake blue, Model 1 with the interaction; Red, Model 2 with the interaction; Black, Model 3 with the interaction), respectively. Solid circles indicate that zero is not included in 95% CIs, suggesting significant relations. Hollow circles indicate that zero is included in 95% CIs, suggesting insignificant relations. Gender was dummy-scored as girl = 0 and boy = 1. For instance, high concentrations of isovalerate predicted less mother-reported externalizing behavior in boys than girls.

Table S1. Internal consistency of the SDQ subscales.

	Subscale	ω_{total} estimate
Child reports	Internalizing	0.74
	Externalizing	0.74
	Prosocial	0.56
Maternal reports	Internalizing	0.77
	Externalizing	0.76
	Prosocial	0.71

Table S2. Food frequency questionnaire items and their loadings in factor analysis.

No.	Item	Factor 1 (healthy foods)	Factor 2 (snacks)
1	Fruit	0.53	-0.04
2	Vegetables	0.41	-0.02
3	Skimmed milk	0.06	-0.02
4	Whole milk	0.08	0.19
5	Cheese	0.27	-0.22
6	Yoghurt	0.58	0.22
7	Ice	0.09	0.26
8	Processed meats	0.08	0.34
9	Other meats	-0.11	0.16
10	Fried fish	0.09	0.20
11	Other fish	0.28	0.16
12	Non-dairy drinks with sugar	-0.32	0.38
13	Non-dairy drinks without sugar	0.03	0.25
14	Breakfast cereals	0.30	0.06
15	White bread	-0.23	0.18
16	Brown bread	0.19	0.04
17	Rice	0.15	0.18
18	Pasta	0.18	0.18
19	Chips and nuts	-0.09	0.57
20	Cafeteria snacks	-0.10	0.36
21	Cake	-0.02	0.35
22	Candy	0.03	0.25
23	Ketchup	0.14	0.17
24	Peanut butter or chocolate spread	-0.04	0.28
25	Jam or honey	0.35	0.07

To reduce the number of food items, we carried out a factor analysis to determine the optimal number of factors. Two factors were the optimal number for the current study. As yoghurt, fruit and vegetables were the main positive contributors to the Factor 1, we named this factor "healthy foods". Chips, nuts, and non-dairy drinks with sugar were the main positive contributors to the Factor 2, thus we named it "snacks". Both factors were used as diet-derived variables in this study.

Table S3. Concentrations of microbiota-derived fecal metabolites with tests for gender-related differences.

Metabolite	Prevalence %	All (Mean ± SD %)	Girls (Mean ± SD %)	Boys (Mean ± SD %)	*p*	*p.adj*
Lactate	82	2.867 ± 3.96	3.036 ± 3.602	2.718 ± 4.268	0.331	0.997
Acetate	100	32.033 ± 14.319	31.779 ± 14.762	32.255 ± 14.019	0.957	0.997
Propionate	100	13.317 ± 5.972	12.805 ± 6.508	13.766 ± 5.465	0.093	0.929
Isobutyrate	86	3.01 ± 4.846	3.095 ± 5.838	2.935 ± 3.811	0.807	0.997
Butyrate	97	8.985 ± 6.572	8.721 ± 5.971	9.217 ± 7.09	0.997	0.997
Isovalerate	91	2.17 ± 1.454	2.155 ± 1.519	2.183 ± 1.406	0.86	0.997
Valerate	72	1.084 ± 0.964	1.062 ± 0.989	1.104 ± 0.948	0.668	0.997
Total SCFAs (including acetate, propionate, and butyrate)	100	58.667 ± 24.437	57.389 ± 25.718	59.787 ± 23.378	0.502	0.997
Total BCFAs (including isobutyrate and isovalerate)	95	5.179 ± 5.344	5.25 ± 6.266	5.117 ± 4.426	0.727	0.997
The ratio of total BCFAs to total SCFAs	-	0.358 ± 0.289	0.367 ± 0.316	0.35 ± 0.266	0.741	0.997

Unit: μmol/gram of wet feces. The prevalence % values indicate the prevalence of metabolites across all participants. The *p* values refer to the significance of Wilcoxon tests between genders before the FDR adjustment, while *p.adj* values indicate the adjusted significance.

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Chapter 5

Development of the gut microbiota in the first 14 years of life and its relations to problem behavior and social anxiety during puberty

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Abstract

Background: Relationships between the gut microbiota and host mental health have been suggested by a growing number of case-control and cross-sectional studies, while supporting evidence is limited in large community samples followed during an extended period. Therefore, we focused on child gut microbiota development in the first 14 years of life and explored its relations to problem behavior and social anxiety in puberty, a period of high relevance for the development of mental health problems.

Results: Fecal microbiota composition was analysed by 16S ribosomal RNA gene amplicon sequencing in a total of 1003 samples from 193 children. Through a clustering method, four distinct microbial clusters were newly identified in puberty. Most children within three of these clusters remained in the same clusters from the age of 12 to 14 years, suggesting stability in microbial development and transition during this period. These three clusters were compositionally similar to enterotypes (i.e., a robust classification of the gut microbiota based on its composition across different populations) enriched in *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively. Two *Prevotella* 9-predominated clusters, including one reported by us earlier in middle childhood and the other one in puberty, were associated with more externalizing behavior at age 14. One *Faecalibacterium*-depleted pubertal cluster was related to more social anxiety at age 14. This finding was confirmed by a cross-sectionally negative relation between *Faecalibacterium* and social anxiety in the 14-year-olds.

Conclusions: The findings of this study continue to map gut microbiota development in a relatively large community sample followed from birth onwards, importantly extending our knowledge to puberty. Results indicate that *Prevotella* 9 and *Faecalibacterium* may be relevant microbial taxa in relation to externalizing behavior and social anxiety, respectively. These correlational findings need validations from other similar cohort studies, as well as well-designed mechanistic pre-clinical investigations before inferring cause and effect.

Keywords: Gut microbiota development; puberty; externalizing behavior; social anxiety; *Prevotella* 9; *Faecalibacterium*.

Introduction

The gut microbiota plays a critical role in many fundamental aspects of health, and its normal development at early ages is required to maintain host fitness during childhood and later in life (Fan & Pedersen, 2021; Margolis, Cryan, & Mayer, 2021). Longitudinal studies have uncovered that the gut microbiota develops in a relatively dynamic pattern in infancy and toddlerhood (Hermes, Eckermann, de Vos, & de Weerth, 2020; Roswall et al., 2021; Stewart et al., 2018). Importantly, this ecological succession may not come to an end within the first three years of life as previously believed (Koenig et al., 2011; Yatsunenko et al., 2012), but changes continue at least till middle childhood, likely as a result of external influential factors (Ou, Belzer, Smidt, & de Weerth, 2022). However, to date it is unclear if microbial community succession processes continue in puberty, a period with large physical, hormonal, emotional, and social changes.

The gut microbiota is tightly involved in mental health and disorders (Cryan et al., 2019). Effects of the bidirectional communication along the microbiota-gut-brain axis (MGBA) are thought to be more profound during sensitive developmental windows, such as infancy and puberty (Cryan et al., 2019; de Weerth, 2017; Margolis et al., 2021). In puberty, children tend to manifest more internalizing and externalizing behavioral problems. Internalizing problem behavior influences the internal psychological environment (withdrawal, anxious, and depressive features), while externalizing problem behavior is exhibited in the external environment (impulsive, aggressive, and hyperactive features) (Liu, 2004). Notably, problem behavior in infancy and middle childhood has been related to gut microbial alpha diversity and relative abundances of individual microbial taxa (Laue et al., 2021; Loughman et al., 2020; Ou et al., 2022; Van De Wouw et al., 2022). Whether similar links exist in puberty remains under-explored till now. In puberty, typically developing children seek to strengthen bonds with their peers and become increasingly independent from their parents (Collins, 1997; Schacter & Margolin, 2019). These changes in child behavior increase the risk of developing social anxiety, a complaint that falls under internalizing behavior and plays an important role as a potential antecedent of other internalizing symptoms, such as depression and loneliness (Hilimire, DeVylder, & Forestell, 2015). Regarding the MGBA, lower alpha diversity levels and higher *Bacteroides* and *Escherichia-Shigella* relative abundances have been reported in patients with generalized anxiety disorder (GAD) (Y. huan Chen et al., 2019; Jiang et al., 2018; Mason et al., 2020), but information on gut microbial links to social anxiety symptoms in community children in puberty is as yet lacking.

Therefore, our first aim was to describe gut microbiota development from birth to puberty in a low-risk longitudinal cohort (N=193 at birth). To this end, pubertal clusters were determined in samples from the ages of 12 and 14 years and combined to the previously determined microbial clusters from infancy (ages one, three, and four months) and middle childhood (ages six and ten years) (Ou et al., 2022). Thereafter, associations between the gut microbiota in the first 14 years of life and problem behavior and social anxiety at age 14 were investigated. The associations were analysed in two ways: (1) relations of microbial clusters and phylogenetic diversity over time with child behavioral measures at age 14; (2) cross-

sectional relations between individual taxon relative abundances and behavioral measures at age 14.

Materials and Methods

Study subjects

The study included fecal samples collected at the ages of one, three, and four months, and six, ten, 12, and 14 years, from an ongoing longitudinal cohort named BIBO (N=193 originally recruited in pregnancy), with approval from the ethical committee of the Faculty of Social Sciences of Radboud University, Nijmegen, the Netherlands (ECG300107, ECG13012012, SW2017-1303-497 and SW2017-1303-498). The original recruitment criteria and procedures are described elsewhere (Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011). The present study was preregistered on the OSF platform (https://osf.io/8ymav).

Data collection procedures

The original process and criteria of recruitment are described elsewhere (Beijers et al., 2011). Data collection procedures till age ten have been described previously (Ou et al., 2022), and the descriptions of data collection at age 12 can be found through the link (https://osf.io/wu2vt). A total of 143 children participated in the round at age 14, of which a number of 125 provided fecal samples. Child characteristics and descriptives as well as their missingness are displayed in Table 1.

Gut microbiota composition

Regarding the fecal samples at age 14, we used the same DNA isolation protocol as used for earlier samples (Ou et al., 2022). In brief, 0.01 to 0.13 g of fecal samples were used for microbial DNA extraction through the Maxwell 16 Total RNA system (Promega, Wisconsin, USA). Duplicate amplicons of the V4 region of bacterial and archaeal 16S ribosomal RNA (rRNA) genes were purified and adjusted to 200 ng per sample prior to being sequenced.

The sequence data in puberty (i.e., N = 139 and 125 samples available at the ages of 12 and 14, respectively) were included and processed using the *NG-Tax* 2.0 pipeline to identify amplicon sequence variants (ASVs) (Poncheewin et al., 2020; Ramiro-Garcia et al., 2018). Those ASVs were taxonomically assigned based on the SILVA_138_SSU 16S rRNA gene reference database (Quast et al., 2012). A total of 52,054,996 reads were obtained, with a median of 182,740 reads per sample. Taxa observed in puberty were used in microbial cluster identification and behavioral relation investigation as outlined below. Regarding microbial data till age ten (i.e., N = 739 samples at ages of one, three, and four months, and six and ten years), we directly used the microbial clusters (i.e., three clusters in infancy and four clusters in middle childhood) and phylogenetic diversity presented in our earlier study (Ou et al., 2022).

Behavioral measures

Children at age 14 were asked to fill in the Strengths and Difficulties Questionnaire (SDQ) for problem behavior (Goodman, 1997) and the Social Anxiety Scale for Adolescents (SAS-A) for their social anxiety complaints (la Greca, Dandes, Wick, Shaw, & Stone, 1988). The SDQ includes internalizing and externalizing subscales. Each subscale includes ten items, scored

on a three-point scale (zero to two), leading to a final score ranging from zero to 20. The SAS-A includes 18 items used for anxiety evaluation and four filler items not used for calculating the score. Each SAS-A item is scored on a five-point scale (one to five), leading to a total social anxiety score ranging from 18 to 90. Higher scores on internalizing and externalizing behavior, and social anxiety reflect more difficulties. These behavioral measures were confirmed to have acceptable internal consistency represented by ω_{total} values (Revelle & Condon, 2019), namely: internalizing = 0.71, externalizing = 0.68, and social anxiety = 0.94, as calculated with the *psych* R package (Revelle, 2021). Internalizing behavior and social anxiety were highly correlated (Spearman's Rho = 0.72, $p < 0.001$), while externalizing behavior was not correlated to internalizing behavior and social anxiety (Spearman's Rho = 0.11 and 0.10, respectively).

Potential covariates

We also measured variables known to be related to the gut microbiota and host behavior, at child age 14: (1) age in years; (2) child gender (boy and girl); (3) tanner stages, including thelarche or testicular development and pubarche (both are self-assessed on a five-point scale, with score one indicating a prepubertal status and score five referring to complete sexual maturity); (4) zBMI calculated with the WHO Growth Reference via the *zscore* R package (Myatt & Guevarra, 2019); (5) whether a child was sick in the week before the home visit (Beijers et al., 2011); (6) whether a child took antibiotics in the past one year (Beijers et al., 2011); (7.1) diet quality, measured by an online self-report questionnaire named *Eetscore* (de Rijk et al., 2022), which assesses the adherence to the Dutch dietary guideline. The total score can range from zero to 160 points, with higher scores representing better adherence to the guideline and hence a generally healthier diet; (7.2) consumption of omega-3 fatty acids; (7.3) consumption of probiotics; (8) physical activity, measured by Physical Activity Questionnaire for Adolescents (PAQ-A) (Kowalski, Crocker, & Kowalski, 1997). The final PAQ-A activity summary score ranges from one to five, with score one indicating low physical activity and score five indicating high physical activity; (9) the use of alcohol, tobacco, and drugs, measured by Brief Screener for Tobacco, Alcohol, and Other Drugs (BSTAD) (Kelly et al., 2014); (10) stool consistency as measured by the seven-point Bristol stool scale, with type one indicating the most lumpy and type seven referring to the most liquid(O'Donnell, Virjee, & Heaton, 1990). Types three and four (i.e., sausage- or snake-like with either cracks on surface or being smooth and soft) are considered as normal stool types in general populations (Heaton et al., 1992); (11) maternal and paternal education levels ranging from one to eight, with higher scores indicating higher levels of education; (12) overnight sleep duration in hours, measured by the Pittsburg Sleep Quality self-report questionnaire (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989); (13) pets (yes or no).

Statistical analyses

All analyses were performed in R studio (version 4.1) with the *phyloseq*, *microbiome*, *picante*, *dplyr*, *data.table*, *tidyr*, *moments*, *faraway*, *gtsummary*, *ComplexHeatmap*, *ggpubr*, *microbiomeMarker*, and *MASS* R packages.

First aim: Gut microbiota development in the first 14 years of life

We used microbial clusters (i.e., conserved compositional patterns of the gut microbiota) to describe gut microbiota development from birth till age 14. Microbial clusters from birth till age ten were identified through Dirichlet multinomial mixture (DMM) models (Holmes, Harris, & Quince, 2012) in our earlier research (Ou et al., 2022), and therefore we directly included them in the current study. Here we analyzed microbial data at ages 12 and 14 together by using the same clustering methods. The optimal number of pubertal microbial clusters was determined by the lowest Laplace approximation score.

Development and transition of pubertal microbial clusters were displayed together with the infant and childhood microbial clusters reported previously. ASV-based phylogenetic diversity and genus-level beta diversity (using weighted UniFrac distance) were compared between pubertal microbial clusters. LEfSe (i.e., Linear discriminant analysis Effect Size) was used to identify differentially abundant microbial taxa between pubertal clusters. Multiple comparisons were corrected with the false discovery rate (FDR) method.

Additionally, we assessed if pubertal microbial clusters were associated with the potential covariates aforementioned. To this end, we used redundancy analysis (RDA) to evaluate to what extent the microbial variance at age 14 was explained by potential covariates. Both simple and marginal effects were measured. Simple effects refer to variance explained by one variable without considering any other variables. Marginal effects mean variance explained by one variable after variance explained by other variables was taken out.

Second aim: Associations between the gut microbiota across the first 14 years of life and behavioral measures at age 14

Generalized linear models (GLMs) were implemented to assess relations of microbial clusters and phylogenetic diversity over time with behavioral measures (i.e., internalizing and externalizing behavior, and social anxiety) at age 14. Additionally, GLMs were also conducted to measure cross-sectional relations between individual taxon relative abundances at the genus level and the behavioral measures at age 14. We also described how much microbial variance at age 14 was explained by behavioral measures at the same age through RDA.

To select the best fitting distributions for behavioral outcomes, we measured their distribution normality and skewness. Internalizing behavior and social anxiety were right-skewed (skewness = 0.97 and 0.60) and non-normally distributed (normality assessed by the Shapiro–Wilk test, $p < 0.01$ for both, indicating non-normal distribution), and therefore negative binomial distribution was used in GLMs (Green, 2021). Externalizing behavior was normally distributed ($p = 0.08 > 0.05$) and not skewed (skewness = 0.12), so the normal distribution was used in GLMs.

Two different models were conducted as follows:

(1) a crude model of $B_i \sim M_j$ was used to measure the independent relation between behavioral measures and microbial parameters. "B_i" represents the matrix of behavioral measures, with "$_i$" referring to either internalizing or externalizing behavior, or social anxiety. "M_j" indicates microbial parameters, with "$_j$" being either microbial clusters, phylogenetic diversity, or relative abundances of an individual genus-level taxon prevalent in more than 10% of 14-year-old samples.

(2) an adjusted model of $B_i \sim M_j$ + *potential covariates* was implemented when its corresponding crude model was found to have an original unadjusted $p < 0.05$. Before conducting adjusted models, we assessed independent relations between the behavioral measures and their potential covariates with GLMs (Table S1). Those with original $p < 0.05$ were used in the adjusted models (Cinelli, Forney, & Pearl, 2020), including: (a) child gender, diet quality, and overnight sleep duration were included for internalizing behavior; (b) overnight sleep duration and alcohol intake were included for externalizing behavior; (c) child gender, diet quality, overnight sleep duration, and paternal education levels were included for social anxiety. The variance inflation factor (VIF) values of M_j and *potential covariates* in all adjusted models were lower than three, indicting no multicollinear issues (Zuur, Ieno, & Elphick, 2010).

Multiple GLM tests were corrected by FDR methods.

Significance

The significance was defined as $p < 0.05$ for non-multiple tests or FDR-adjusted $p < 0.05$ for multiple tests.

Results

Population characteristics and descriptives

Approximately half of the children participating in the round of age 14 were boys (Table 1). Compared to boys, girls developed significantly quicker in sexual maturity and had more self-reported internalizing behavior and social anxiety. Furthermore, girls exhibited insignificant but slightly higher zBMI values, better diet quality, lower Bristol scores, and fewer sleeping hours (unadjusted $p < 0.10$). Regarding microbial variance explained by potential covariates (Table S2), overnight sleep duration accounted for 3.05% total variation (simple effect, $p < 0.01$), followed by drinking alcohol (simple effect, $R^2\% = 1.72\%$ but insignificant with $p = 0.07$). The significance remained for overnight sleep duration after partitioning the variance explained by other variables (marginal effect, $R^2\% = 2.07\%$ and $p = 0.03$).

Table 1. Population characteristics and descriptives at age 14.

Variable	Overall, N = 143[1]	Boy, N = 77[1]	Girl, N = 66[1]	p-value[2]	Adjusted p-value[3]
Age in years	14.46 (0.17)	14.46 (0.16)	14.45 (0.19)	0.4	0.7
Thelarche or testicular development	3.81 (0.78)	3.69 (0.80)	3.95 (0.73)	0.044	0.2
Pubarche	3.64 (0.85)	3.35 (0.74)	3.97 (0.86)	<0.001	<0.001
zBMI	-0.09 (1.00)	-0.18 (1.04)	0.02 (0.95)	0.093	0.2
Sick in the week before the home visit (yes/overall)	7 / 138 (5.1%)	3 / 73 (4.1%)	4 / 65 (6.2%)	0.7	0.8
(Missing)	5	4	1		
Oral antibiotics in the past one year (yes/overall)	2 / 143 (1.4%)	0 / 77 (0%)	2 / 66 (3.0%)	0.2	0.5
Diet quality	86.94 (16.84)	84.06 (17.74)	90.15 (15.29)	0.079	0.2
(Missing)	12	8	4		
Omega-3 fatty acids (yes/overall)	5 / 138 (3.6%)	2 / 73 (2.7%)	3 / 65 (4.6%)	0.7	0.8
(Missing)	5	4	1		
Probiotics (yes/overall)	0 / 138 (0%)	0 / 73 (0%)	0 / 65 (0%)	-	-
(Missing)	5	4	1		
Physical activity	2.34 (0.55)	2.33 (0.57)	2.34 (0.54)	>0.9	>0.9
(Missing)	7	5	2		
Drinking alcohol in the past one year (yes/overall)	29 / 141 (21%)	17 / 75 (23%)	12 / 66 (18%)	0.5	0.7
(Missing)	2	2	0		
Smoking cigarettes in the past one year (yes/overall)	4 / 141 (2.8%)	1 / 75 (1.3%)	3 / 66 (4.5%)	0.3	0.6
(Missing)	2	2	0		
Taking drugs in the past one year (yes/overall)	5 / 141 (3.5%)	2 / 75 (2.7%)	3 / 66 (4.5%)	0.7	0.8
(Missing)	2	2	0		
Bristol score[4]	3.12 (0.96)	3.23 (0.91)	2.98 (1.00)	0.058	0.2
(Missing)	23	13	10		
Maternal education level	5.94 (1.33)	5.79 (1.51)	6.12 (1.06)	0.4	0.7
Paternal education level	5.39 (1.83)	5.34 (1.95)	5.45 (1.69)	>0.9	>0.9
(Missing)	8	4	4		

Overnight sleep duration in hours	8.24 (1.06)	8.37 (1.12)	8.10 (0.99)	0.086	0.2
(Missing)	6	5	1		
Pets (yes/overall)	96 / 143 (67%)	49 / 77 (64%)	47 / 66 (71%)	0.3	0.6
Internalizing behavior	4.14 (3.03)	3.02 (2.39)	5.44 (3.20)	<0.001	<0.001
(Missing)	1	1	0		
Externalizing behavior	6.33 (2.94)	6.16 (3.09)	6.52 (2.76)	0.7	0.8
(Missing)	1	1	0		
Social anxiety	41.20 (12.62)	38.14 (12.17)	44.63 (12.32)	0.001	0.008
(Missing)	5	4	1		

[1]Mean (SD); n / N (%)

[2]Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test

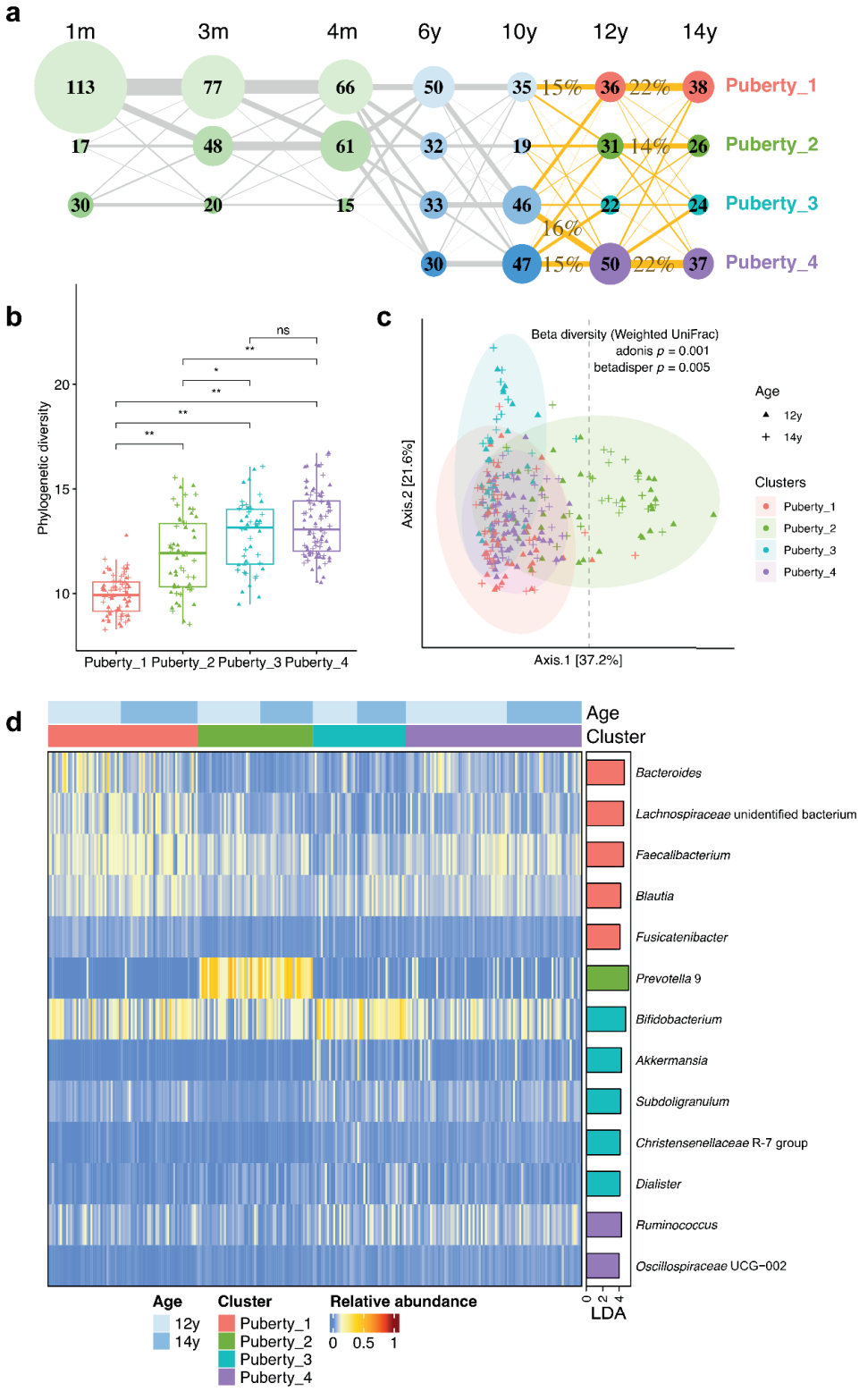
[3]False discovery rate correction for multiple testing

[4]Bristol stool consistency scale was used as a numeric variable here. The distribution of Bristol stool consistency types in categorical format is displayed in Figure S1.

Gut microbiota development in the first 14 years of life

Microbial clusters and their transition

Four microbial clusters were identified from N = 264 pubertal samples at the ages of 12 and 14 years based on their compositional features (Figure 1a), determined by the lowest Laplace value in DMM models (Figure S2). No significant differences were observed in potential covariates between these clusters after FDR corrections (Table S3). However, Puberty_2 and Puberty_4 tended to include more boys (67%, 38/57 boys in Puberty_2; 60%, 52/87 boys in Puberty_4), and Puberty_3 consisted of fewer boys (37%, 17/46); Pearson's Chi-squared test p = 0.009 and adjusted p = 0.2. Besides, children within Puberty_1 likely took more oral antibiotics (8.1%, 6/74 had oral antibiotics in Puberty_1, and less than 5% in the other three clusters); Fisher's exact test p = 0.034 and adjusted p = 0.2. Furthermore, 38% (10/26) of 14-year-old children within Puberty_2 drank alcohol in the past one year, which was more frequent than those belonging to other pubertal clusters at this age (less than 20%); Fisher's exact test p = 0.016 and adjusted p = 0.2.


a
1m
3m
4m
6y
10y
12y
14y
113
77
66
50
35
15%
36
22%
38
Puberty_1
17
48
61
32
19
31
14%
26
Puberty_2
30
20
15
33
46
22
24
Puberty_3
16%
30
47
15%
50
22%
37
Puberty_4
b
Phylogenetic diversity
20
15
10
ns
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Puberty_1 Puberty_2 Puberty_3 Puberty_4
c
Beta diversity (Weighted UniFrac)
adonis p = 0.001
betadisper p = 0.005
Axis.2 [21.6%]
Axis.1 [37.2%]
Age
12y
14y
Clusters
Puberty_1
Puberty_2
Puberty_3
Puberty_4
d
Age
Cluster
Bacteroides
Lachnospiraceae unidentified bacterium
Faecalibacterium
Blautia
Fusicatenibacter
Prevotella 9
Bifidobacterium
Akkermansia
Subdoligranulum
Christensenellaceae R-7 group
Dialister
Ruminococcus
Oscillospiraceae UCG-002
0
2
4
LDA
Age
12y
14y
Cluster
Puberty_1
Puberty_2
Puberty_3
Puberty_4
Relative abundance
0
0.5
1

Figure 1. Microbial clusters. (a) Transition between microbial clusters in the first 14 years of life. Microbial clusters were determined by the DMM clustering method according to their compositional characteristics at the genus level. The clusters in infancy (i.e., ages of one, three, and four months) and middle childhood (i.e., ages of six and ten years) were reported previously (Ou et al., 2022), and the clusters in puberty (i.e., ages of 12 and 14 years) were determined in the present study. Microbial clusters are presented as nodes, with the size and the number indicating how many samples belong to the corresponding cluster. The four pubertal clusters are colored in pink, grass green, lake blue, and purple, respectively. Transition rates between clusters are shown as sized lines. The lines from ages ten to 14 are highlighted in orange, accompanied with transition rates (>10%) in percentages. (b) Phylogenetic diversity of pubertal microbial clusters. Wilcoxon tests were implemented between clusters with the FDR correction (adjusted *p*: ns, not significant; *, <0.05; **, < 0.01). (c) Beta diversity between pubertal microbial clusters. It was calculated by weighted UniFrac distance based on relative abundance data of genus-level microbial taxa. (d) Differentially abundant genus-level taxa between microbial clusters in puberty. These taxa were identified through LEfSe (Linear discriminant analysis Effect Size) with FDR-adjusted $p < 0.05$ and LDA (Linear discriminant analysis) effect size > 4. Taxon relative abundances in individuals are shown in the heatmap on the left side. The barplot on the right side represents LDA scores, with colors indicating enriched clusters.

At age 12, 26%, 22%, 16%, and 36% (36, 31, 22, and 50/139) of children belonged to microbial clusters Puberty_1, Puberty_2, Puberty_3, and Puberty_4, respectively. At age 14, proportions of Puberty_1 and Puberty_3 increased (30% and 19%; 38 and 24/125, respectively), while ratios of Puberty_2 and Puberty_4 decreased (21% and 30%; 26 and 37/125, respectively).

In puberty, N = 116 children provided both samples at the ages of 12 and 14. Of these children, 22% remained in Puberty_1, another 22% remained in Puberty_4, and 14% remained in Puberty_2. In contrast, children within Puberty_3 at age 12 showed a more diverse developing track from ages 12 to 14. Among N = 130 completed cases at the ages of ten and 12, 16% and 15% of these children transitioned from childhood microbial clusters 3 and 4 to Puberty_4, while 15% of them developed from childhood cluster 1 to Puberty_1.

Compositional features of pubertal microbial clusters

Puberty_1 showed the lowest phylogenetic diversity followed by Puberty_2, and Puberty_3 and Puberty_4 exhibiting the highest phylogenetic diversity (Figure 1b). Besides, we observed different compositional variances (i.e., heterogeneity of dispersion) among pubertal clusters (betadisper $p = 0.005$; Figure 1c). Specifically, Puberty_4 differed from Puberty_2 and Puberty_3 (betadisper $p = 0.001$ for both). The adonis function ($p = 0.001$) further showed general dissimilarity in microbial composition between pubertal clusters. Pairwise comparisons between clusters confirmed this result (adonis $p = 0.001$ for all). Additionally, we found 31 samples within Puberty_2 (including N = 14 at age 12 and N = 17 at age 14) located dispersedly (as shown on the right side of the vertical dashed line), in comparison with other samples in puberty.

Nine, 15, 28, 43, and 105 microbial taxa were found differentially abundant between pubertal clusters at the levels of phylum, class, order, family, and genus, based on LEfSe analysis (effect size > 2 and FDR-adjusted $p < 0.05$), respectively. Particularly, Puberty_1 was enriched in *Bacteroides*, an unidentified genus within *Lachnospiraceae* family,

Faecalibacterium, *Blautia*, and *Fusicatenibacter*, Puberty_2 was predominated by *Prevotella* 9, Pubety_3 was enriched in *Bifidobacterium*, *Akkermansia*, *Subdoligranulum*, *Christensenellaceae* R-7 group, and *Dialister*, and Puberty_4 was enriched in *Ruminococcus* and *Oscillospiraceae* UCG-002 (Figure 1d).

Associations between the gut microbiota across the first 14 years of life and behavioral measures at age 14

Relations of microbial clusters and phylogenetic diversity over time with child behavioral measures at age 14

First, independent relations between microbial predictors at each time point or period (i.e., either microbial clusters or phylogenetic diversity in infancy including one, three, and four months, childhood including six and ten years, or puberty including 12 and 14 years) and behavioral outcomes at age 14 (i.e., internalizing and externalizing behavior, and social anxiety) were determined by crude generalized linear models (GLMs), without accounting for any covariates. Next, we adjusted GLMs with potential covariates for the behavioral outcomes. This was based on covariates that displayed original *p* values lower than 0.05 in crude GLMs (Table 2; See detailed GLM results regarding clusters and phylogenetic diversity in Table S4 and Table S5, respectively).

Table 2. Main findings of the relations between either microbial clusters or phylogenetic diversity in the first 14 years of life and behavioral outcomes at age 14.

Age	Cluster or diversity	Crude model			Adjusted model			
		Estimate (Std. Error)	*p*	Adjusted *p*	Estimate (Std. Error)	*p*	Adjusted *p*	VIF
Internalizing behavior								
Infancy	Infancy_2	0.2 (0.1)	0.044	0.102	0.2 (0.1)	0.007	0.021	1.02
Infancy	Phylogenetic diversity	<0.1 (<0.1)	0.016	0.030	<0.1 (<0.1)	0.002	0.004	1.03
Externalizing behavior								
6y	Childhood_2	1.9 (0.7)	0.008	0.023	1.6 (0.7)	0.025	0.060	1.13
12y	Puberty_2	1.9 (0.7)	0.012	0.033	1.7 (0.7)	0.021	0.054	1.06
14y	Puberty_2	1.8 (0.7)	0.012	0.034	1.0 (0.7)	0.190	0.308	1.18
Childhood	Childhood_2	1.7 (0.5)	0.002	0.009	1.4 (0.5)	0.008	0.023	1.07
Puberty	Puberty_2	1.9 (0.5)	<0.001	0.001	1.3 (0.5)	0.010	0.029	1.08
Social anxiety								
3m	Infancy_2	0.1 (0.1)	0.033	0.078	0.2 (0.1)	0.003	0.012	1.08
14y	Puberty_3	0.2 (0.1)	0.017	0.044	0.2 (0.1)	0.015	0.040	1.17
Puberty	Puberty_3	0.1 (0.1)	0.048	0.107	0.2 (0.1)	0.004	0.012	1.11

Notes. Only clusters or phylogenetic diversity with original $p < 0.05$ in crude GLMs are displayed here. As microbial cluster is a categorical variable, comparisons were implemented between the first cluster and other clusters at the corresponding time point or period. Phylogenetic diversity was used as a numeric variable. In adjusted models, child gender, diet quality, and overnight sleep duration were included for internalizing behavior; overnight sleep duration and alcohol intake were included for externalizing behavior; and child gender, diet quality, overnight sleep duration, and paternal education levels were included for social anxiety. VIF < 3 indicates no multicollinearity in adjusted models.

In adjusted GLMs, we observed more internalizing behavior in cluster Infancy_2 in the period from ages one to four months (estimate = 0.2 and adjusted $p = 0.021$), but not at separate time points. Similarly, we found more externalizing behavior in Childhood_2 and Puberty_2 during their corresponding periods in adjusted GLMs (estimates = 1.4 and 1.3, respectively; adjusted $p = 0.023$ and 0.029, respectively). Besides, more social anxiety was found in Infancy_2 at the age of three months and Puberty_3 at the age of 14 years and in the

period of puberty (estimates ≤ 0.2 and adjusted $p < 0.05$ after accounting for covariates). With respect to phylogenetic diversity, the only significant finding was observed in infancy, with a mildly positive relation to social anxiety at age 14 (estimate < 0.1; adjusted $p = 0.004$ in the adjusted GLM).

Additionally, we explored differences in behavioral relations between disperse Puberty_2 samples and other samples in puberty based on beta diversity (Table S6). To this end, we performed the same crude and adjusted GLMs described above. Disperse Puberty_2 samples at age 14 showed significantly more internalizing behavior at the same age without accounting for covariates (estimate = 0.4 and adjusted $p = 0.02$), while the difference became marginally significant after considering covariates (estimate = 0.3 and adjusted $p = 0.079$). Similarly, after partialling out potential influences of covariates, disperse Puberty_2 samples in the period of puberty did not exhibit more externalizing behavior (crude GLM: estimate = 1.3 and adjusted $p = 0.041$; adjusted GLM: estimate = 0.6 and adjusted $p = 0.338$).

Cross-sectional relations between the gut microbiota and behavioral measures in 14-year-old children

RDAs showed that externalizing behavior was the only behavioral measure that significantly explained microbial variance in the 14-year-olds without considering other variables (simple effect, $R^2\% = 1.93\%$ and $p = 0.04$; Table S2). However, after partitioning the variance explained by overnight sleep duration and drinking alcohol, externalizing behavior did not remain significant (marginal effect, $R^2\% = 0.58\%$ and $p = 0.71$). We further measured cross-sectional relations between relative abundances of individual genus-level taxa and the behavioral measures at age 14. Table 3 presents the results of taxa in which the original significance in crude GLMs was $p < 0.05$.

Table 3. Main findings of the relations between taxon relative abundances at the genus-level and behavioral measures in children at age 14.

	Crude model			Adjusted model				Fold change of estimates (crude / adjusted)	Mean of relative abundance (SD) %	Prevalence %
Genus	Estimate (Std. Error)	*p*	Adjusted *p*	Estimate (Std. Error)	*p*	Adjusted *p*	VIF			
Internalizing behavior										
Agathobacter	81.1 (34.4)	0.020	0.038	49.3 (32.5)	0.132	0.212	1.02	1.65	0.1 (0.2)	44.8
Barnesiella	-28.4 (12.3)	0.023	0.044	-20.3 (11.1)	0.071	0.123	1.02	1.40	0.4 (0.5)	70.4
Collinsella	-9.6 (4.7)	0.045	0.083	-4.1 (4.3)	0.340	0.455	1.07	2.34	0.7 (1.4)	52.0
Faecalibacterium	-3.1 (1.2)	0.012	0.023	-2.2 (1.2)	0.073	0.126	1.12	1.41	9.3 (5.0)	99.2
Intestinibacter	17.5 (8.4)	0.039	0.073	14.6 (7.7)	0.060	0.106	1.01	1.20	0.6 (0.7)	72.8
Lachnospira	36.4 (15.0)	0.017	0.033	18.9 (18.4)	0.305	0.421	1.07	1.93	0.3 (0.4)	57.6
Turicibacter	31.8 (13.7)	0.022	0.043	26.0 (12.5)	0.039	0.073	1.01	1.22	0.2 (0.4)	32
Externalizing behavior										
Erysipelatoclostridium	-648.0 (288.8)	0.027	0.051	-649.8 (276.0)	0.020	0.039	1.00	1.00	<0.1 (0.1)	15.2
Holdemanella	51.2 (15.2)	0.001	0.002	45.8 (14.7)	0.002	0.005	1.01	1.12	0.7 (1.6)	24.8
Lachnospiraceae ND3007 group	-72.5 (26.4)	0.007	0.014	-55.5 (28.5)	0.054	0.097	1.11	1.31	0.9 (1.0)	90.4
Oscillospiraceae NK4A214 group	81.6 (27.0)	0.003	0.006	67.2 (26.2)	0.012	0.023	1.02	1.21	0.6 (0.9)	84.8
Phascolarctobacterium	55.8 (21.2)	0.010	0.019	54.8 (20.6)	0.009	0.018	1.00	1.02	0.6 (1.2)	34.4
Prevotella 9	4.2 (2.0)	0.038	0.071	1.8 (2.0)	0.388	0.507	1.11	2.33	7.2 (12.9)	44.8
Eubacterium uncultured bacterium	57.1 (26.2)	0.031	0.059	52.3 (25.4)	0.042	0.077	1.01	1.09	1.0 (1.0)	79.2
Social anxiety										
Collinsella	-4.6 (1.8)	0.012	0.024	-3.7 (1.8)	0.045	0.083	1.07	1.24	0.7 (1.4)	52.0
Erysipelatoclostridium	80.5 (28.1)	0.005	0.010	73.8 (30.0)	0.016	0.030	1.02	1.09	<0.1 (0.1)	15.2
Faecalibacterium	-2.2 (0.5)	<0.001	<0.001	-1.8 (0.5)	0.001	0.002	1.10	1.22	9.3 (5.0)	99.2
Lachnospiraceae ND3007 group	-7.3 (2.8)	0.010	0.020	-5.7 (3.0)	0.060	0.107	1.13	1.28	0.9 (1.0)	90.4
Turicibacter	13.4 (5.9)	0.026	0.049	8.6 (6.1)	0.160	0.251	1.03	1.56	0.2 (0.4)	32.0

Notes. Only microbial taxa, of which the original $p < 0.05$ in crude GLMs, are displayed here. In adjusted models, child gender, diet quality, and overnight sleep duration were included for internalizing behavior; overnight sleep duration and alcohol intake

were included for externalizing behavior; and child gender, diet quality, overnight sleep duration, and paternal education levels were included for social anxiety. VIF < 3 indicates no multicollinearity in adjusted models.

In crude GLMs, we found higher relative abundances of *Agathobacter*, *Lachnospira*, and *Turicibacter* in relation to more internalizing problems, while higher relative abundances of *Barnesiella* and *Faecalibacterium* were associated with less internalizing behavior (adjusted $p < 0.05$). However, none of them were significant after considering covariates.

With respect to externalizing behavior, in crude GLMs, we observed that higher relative abundances of *Holdemanella*, *Oscillospiraceae* NK4A214 group, *Phascolarctobacterium* were related to more externalizing behavior, while higher relative abundances of *Erysipelatoclostridium* and *Lachnospiraceae* ND3007 group were associated with fewer externalizing issues (adjusted $p < 0.05$, except for *Erysipelatoclostridium* with an adjusted $p = 0.051$). After accounting for covariates and multiple tests, the significance remained for *Holdemanella*, *Oscillospiraceae* NK4A214 group, and *Phascolarctobacterium*. Moreover, the estimate turned into significance for *Erysipelatoclostridium* in the adjusted model (adjusted $p = 0.039$ but with a low average relative abundance < 0.1%). Despite the insignificance, we noticed that the relation between *Prevotella* 9 and externalizing behavior changed strikingly after correcting for covariates (estimate from 4.2 to 1.8 with a fold change = 2.33).

Regarding social anxiety in crude GLMs, positive relations were observed for *Erysipelatoclostridium* and *Turicibacter*, while negative associations were found for *Collinsella*, *Faecalibacterium*, and *Lachnospiraceae* ND3007 (adjusted $p < 0.05$). After adjusting GLMs with covariates, differences remained significant for *Erysipelatoclostridium* and *Faecalibacterium*. Note that *Faecalibacterium* was highly prevalent across 14-year-old children (99.2%) with an average relative abundance at 9.3%.

Discussion

In this study, we focused on a community sample of children in a longitudinal birth cohort (followed from one month to 14 years). Through the DMM method, we identified four distinct microbial clusters among these children in puberty, extending our knowledge on gut microbiota development and transition in this sensitive time window. By including microbial clusters determined in the first decade of life (Ou et al., 2022), we found that two *Prevotella* 9-enriched microbial clusters (i.e., Chilhood_2 and Puberty_2) were related to more externalizing behavior at age 14. Furthermore, Puberty_3, which was characterized by less *Faecalibacterium* compared to *Faecalibacterium*-enriched Puberty_1, was associated with more social anxiety at age 14. Additionally, higher relative abundances of *Faecalibacterium* were cross-sectionally linked to less social anxiety at age 14, supporting the Puberty_3 findings.

Our results indicated some similarities between microbial clusters in middle childhood and puberty (Ou et al., 2022). Puberty_1 resembled Childhood_1 and similarly showed low phylogenetic diversity. Puberty_2 was predominated by *Prevotella* 9, and this

was also a notable feature of Childhood_2. Furthermore, high phylogenetic diversity was observed in Puberty_3 and Puberty_4, seemingly in conformity with Childhood_3 and Childhood_4. Compared to the dynamic succession of microbial clusters in the first decade of life, the transition between pubertal clusters was steadier in this group of children. From the age of 12 to 14 years, most children within Puberty_1, Puberty_2, and Puberty_4 remained in the same clusters. Importantly, these three clusters were enriched in *Bacteroides*, *Prevotella* 9, and *Ruminococcus*, respectively, and this fits well with the three enterotypes reported in 2011, which seemed independent of age across different populations (Arumugam et al., 2011). Therefore, it is possible that Puberty_1, Puberty_2, and Puberty_4 represent a more mature stadium of the gut microbiota. Conversely, Puberty_3 might correspond to a less mature phase, as its transition from age 12 was relatively divergent (i.e., the transition was almost equally towards Puberty_1, Puberty_2, and Puberty_3, indicating the presence of a more unstable cluster without a dominant transitional pattern).

Despite the weak differences between the genders in pubertal microbial clusters (i.e., the differences did not survive FDR corrections), some of these differences appear worth noting. For example, Pubety_2 and Puberty_4 tended to have more boys, while Puberty_3 tended to have more girls. Puberty_3 was enriched in *Bifidobacterium* with β-glucuronidase activity, able to deconjugate inactive bound estrogen into active non-bound estrogen (Yoon & Kim, 2021). Deconjugated estrogen can be reabsorbed by the gut and circulate in the bloodstream. After being conjugated by the liver, a portion of inactive estrogen reaches the gut and in turn may likely affect microbiota composition (Valeri & Endres, 2021). Estrogen, together with androgen, triggers the natural process of sexual maturation in puberty (Emmanuel & Bokor, 2022). It has been suggested that gut microbiota composition may differ between disparate pubertal stages in a gender-dimorphic pattern (Korpela et al., 2021; Yuan, Chen, Zhang, Lin, & Yang, 2020). However, such discrepancy was not observed in our study, which considered pubertal status alone but not its interaction with child gender. Another unexpected finding was that general diet did not appear to explain the different pubertal clusters, while alcohol consumption did. At age 14, *Prevotella* 9-predominant Puberty_2 showed a higher ratio of consuming alcohol. This was in line with a recent finding that increased alcohol consumption, even moderate, was related to higher relative abundances of *Prevotella* 9 in adult populations (Kwan et al., 2022). Given the fact that sample size shrank after stratifying 14-year-old children based on microbial clusters and alcohol intake, our findings must be validated with another larger group of matched children.

Regarding microbial relations to behavioral measures, children within *Prevotella* 9-predominant Childhood_2 and Puberty_2 clusters exhibited more externalizing behavior at the age of 14 years. Although a positive cross-sectional relation was not observed between *Prevotella* 9 and externalizing behavior at age 14 after accounting for alcohol consumption and overnight sleep duration, such a trend conformed to our earlier findings in middle childhood (Ou et al., 2022). In line with this, children with ADHD (attention deficit hyperactivity disorder), who are often characterized by impulsive and hyperactive externalizing symptoms, showed an overgrowth of *Prevotella* species including *P. amnii*, *P. buccae*, and *P. copri*, in comparison with typically developing children (Li et al., 2022). In

particular, higher relative abundances of *P. buccae* were related to more impulsivity and hyperactivity problems, despite another study reporting less *Prevotella* in children with ADHD (Prehn-Kristensen et al., 2018). Other findings which indirectly support links between *Prevotella* and behavior in the externalizing range, are those reporting a *Prevotella* depletion in one-year-old infants with more subsequent inward-directed behavioral issues at two years of age (Loughman et al., 2020), and in GAD-active (generalized anxiety disorder) adults compared to healthy controls (Y. huan Chen et al., 2019). Furthermore, many ASD (autism spectrum disorder) cases show reductions in *Prevotella*, as concluded in a recent systematic review (Bundgaard-Nielsen et al., 2020), while youth with early life adversity (ELA) display higher relative abundances of *Prevotella* (Reid et al., 2021). Before drawing any firm conclusions, we have to be aware of the wide species- and strain-level variability in *Prevotella*, which to a large extent may obscure the consistency between studies (Tett, Pasolli, Masetti, Ercolini, & Segata, 2021). Moreover, potential influences of covariates (e.g., age, gender, diet, and lifestyle) and different etiologies behind mental problems should be considered carefully when comparing results.

More social anxiety was observed in microbial cluster Puberty_3, mainly at the age of 14 years. However, the most enriched taxa in this cluster (i.e., *Bifidobacterium*, *Akkermansia*, *Subdoligranulum*, *Christensenellaceae* R-7 group, and *Dialister*) were not cross-sectionally related to social anxiety at age 14. Nevertheless, higher *Bifidobacterium* has been frequently reported in MDD (major depressive disorder) (Simpson et al., 2021), and lower *Subdoligranulum* and *Dialister* were found in GAD (Y. huan Chen et al., 2019; Jiang et al., 2018), compared to healthy controls. When looking into other taxa, we found that lower *Faecalibacterium*, which was less enriched in Puberty_3 and highly prevalent at age 14, was associated with more social anxiety difficulties, in line with the finding of Puberty_3. Similarly, decreased *Faecalibacterium* has been observed in GAD patients (Jiang et al., 2018), and related to increased duration and intensity of social exclusion experiences in young adults (Kim et al., 2022). Furthermore, recent meta-analytic research described reduced *Faecalibacterium* in multiple mental disorders (L. L. Chen et al., 2021; Simpson et al., 2021), such as MDD, bipolar disorder, and ASD, despite a conflicting ASD result reported by another meta-analytic study (Iglesias-Vázquez, Van Ginkel Riba, Arija, & Canals, 2020). As a gut commensal bacterium, *Faecalibacterium* is present in more than 90% of individuals in adult populations (De Filippis, Pasolli, & Ercolini, 2020). Its most studied and abundant species, *Faecalibacterium prausnitzii,* can produce anti-inflammatory molecules represented by butyrate (Leylabadlo et al., 2020). Apart from regulation of inflammation, butyrate may suppress food intake and mediate cognition by influencing the concentrations of gut hormones (O'Riordan et al., 2022). Taken together, these findings suggest that *Faecalibacterium* may constitute a potentially important microbial marker for mental health.

A strength of our preregistered study is the use of a unique longitudinal community cohort followed from birth till age 14 years. This allows tracking gut microbiota development throughout infancy and childhood, assessing its predictive value for relevant behavioral measures in puberty. Importantly, we simplified the complex interplay between the gut microbiota and behavior by condensing the taxonomic data into identifiable microbial

clusters. Furthermore, this study accounted for multiple potential covariates of behavioral measures when exploring their relations to the gut microbiota, decreasing the correlational bias to some extent. However, some limitations and perspectives should also be mentioned. First, the study was restricted by not considering the interaction of child gender with pubertal stages, mainly due to insufficient statistical power to further stratify groups. Second, although a collection of covariates was included, the gut microbiota and host behavior can still be affected by many unobserved or even unknown variables. Especially for an observational study, it is hence necessary to further validate the findings in another longitudinal community cohort or in carefully designed animal experiments. Third, it remains a statistical challenge to explore relations between repeatedly measured microbiota data and a continuous numeric outcome variable. Currently, statistically sophisticated approaches to identify differentially abundant taxa over time were mainly created for group comparisons (Kodikara, Ellul, & Lê Cao, 2022). Future research should aim to profile microbial trajectories across time and identify distinct ones (Hejblum, Skinner, & Thiébaut, 2015), that can then be linked to host outcome phenotypes, or preferably, to host phenotypical development. Despite recent attempts at describing gut microbiota development, the step of associating variations in trajectories to host behavioral phenotypes is yet to be taken (Roswall et al., 2021). A final limitation of our study lies in the fact that 16S rRNA gene amplicon sequences are unable to provide results at the microbial species level.

Summarizing, in the current study, we identified four distinct microbial clusters in puberty, three of which were compositionally similar to enterotypes previously described at population level across different ages (Arumugam et al., 2011) and transitioned stably from age 12 to 14. Child gender may be a factor driving the formation of microbial clusters in puberty, although we did not find much evidence supporting this idea. The *Prevotella* 9-predominated clusters, including Childhood_2 and Puberty_2, were related to more externalizing behavior at age 14, while the *Faecalibacterium*-depleted Puberty_3 cluster was associated with more social anxiety at the same age. The cross-sectional negative relation between *Faecalibacterium* and social anxiety in 14-year-old children further supported this finding. Causal associations were not determined in this observational longitudinal study. Mechanistic research on a single taxon or an interactive group of taxa is needed to make it possible to describe causal relations between the gut microbiota and child pubertal mental health.

Data availability statement

As the findings in this study are supported by datasets from an ongoing longitudinal cohort, these datasets currently cannot be made publicly available but are available upon request from C.deW. (Carolina.deWeerth@radboudumc.nl)

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Supplemental information

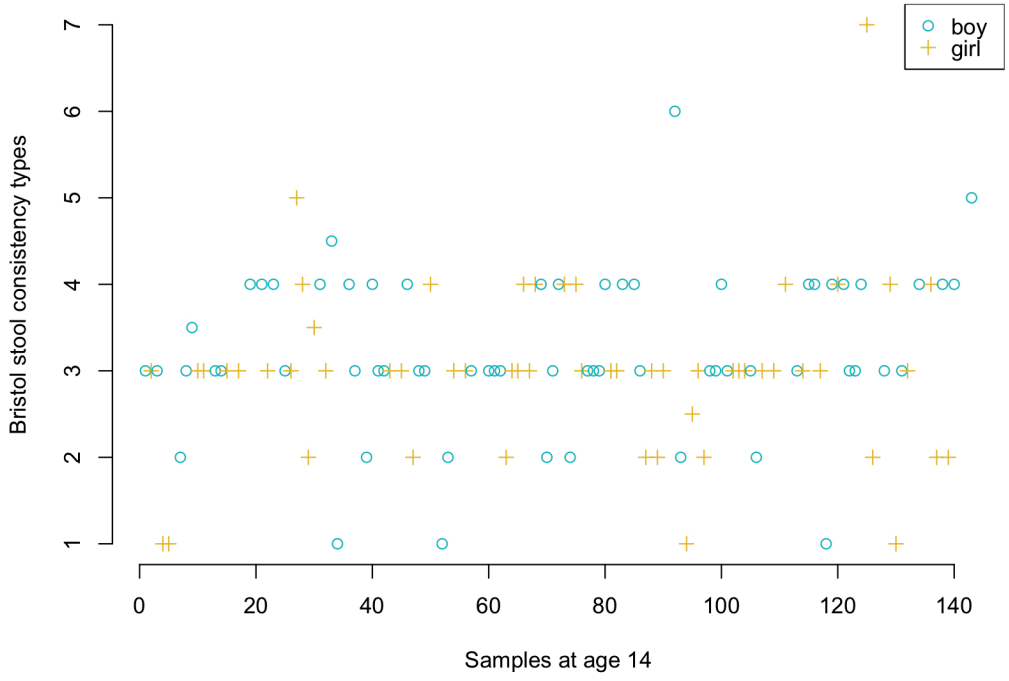

Figure S1. The distribution of Bristol stool consistency types of the samples at age 14. Among N = 125 participants providing stool samples at age 14, N = 7 (boy: girl = 3: 4) reported type 1 stools (separate hard lumps like nuts) during collection, N = 16 (boy: girl = 7: 9) reported type 2 stools (sausage shaped but lumpy), N = 1 girl reported a stool between types 2 and 3 (like a sausage or snake but with cracks on its surface), N = 58 (boy: girl = 29: 29) reported type 3 stools, N = 2 (boy: girl = 1: 1) reported stools between types 3 and 4 (like a sausage or snake, smooth and soft), N = 31 (boy: girl = 21: 10) reported type 4 stools, N = 1 boy reported a stool between types 4 and 5 (soft blobs with clear cut edges), N = 2 (boy: girl = 1: 1) reported type 5 stools, N = 1 boy reported a type 6 stool (fluffy pieces with ragged edges, a mushy stool), and N = 1 girl reported a type 7 stool (watery, no solid pieces).

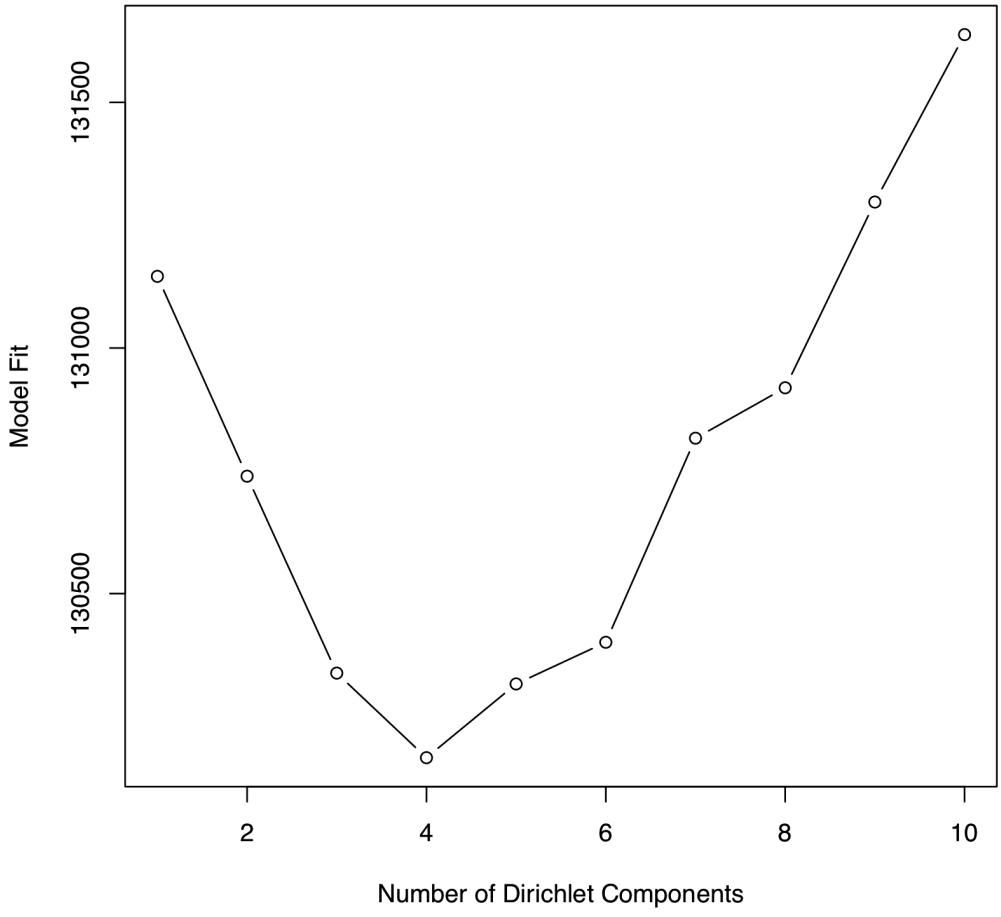

Figure S2. The number of Dirichlet components. Dirichlet Multinomial Mixtures models were conducted N = 10 times. Eighty percent (8/10) of the models showed consistent bacterial clusters of which the optimal number was four, as indicated by the lowest Laplace value.

Table S1. Independent relations between behavioral measures and their potential covariates at age 14.

Behavior	Model	Potential covariate	Parameter	Estimate (Std. Error)	p	Adjusted p
Internalizing behavior	Crude	Age in years	Intercept	6.7 (5.2)	0.197	0.305
Internalizing behavior	Crude	Age in years	Age in years	<0.1 (0.4)	0.309	0.439
Internalizing behavior	Crude	Child gender: girl	Intercept	1.1 (0.1)	<0.001	<0.001
Internalizing behavior	***Crude***	***Child gender: girl***	***Child gender: girl***	***0.6 (0.1)***	***<0.001***	***<0.001***
Internalizing behavior	Crude	Thelarche or testicular development	Intercept	1.6 (0.3)	<0.001	<0.001
Internalizing behavior	Crude	Thelarche or testicular development	Thelarche or testicular development	<0.1 (0.1)	0.523	0.616
Internalizing behavior	Crude	Pubarche	Intercept	1.0 (0.3)	<0.001	0.001
Internalizing behavior	Crude	Pubarche	Pubarche	0.1 (0.1)	0.121	0.198
Internalizing behavior	Crude	zBMI	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	zBMI	zBMI	<0.1 (0.1)	0.691	0.739
Internalizing behavior	Crude	Sick in the week before the home visit: yes	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Sick in the week before the home visit: yes	Sick in the week before the home visit: yes	0.2 (0.3)	0.38	0.494
Internalizing behavior	Crude	Oral antibiotics in the past one year: yes	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Oral antibiotics in the past one year: yes	Oral antibiotics in the past one year: yes	<0.1 (0.6)	0.374	0.492
Internalizing behavior	Crude	Diet quality	Intercept	0.6 (0.3)	0.062	0.109
Internalizing behavior	***Crude***	***Diet quality***	***Diet quality***	***<0.1 (<0.1)***	***0.019***	***0.035***
Internalizing behavior	Crude	Omega 3 fatty acids: yes	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Omega 3 fatty acids: yes	Omega 3 fatty acids: yes	0.4 (0.3)	0.226	0.339
Internalizing behavior	Crude	Physical activity	Intercept	1.8 (0.3)	<0.001	<0.001
Internalizing behavior	Crude	Physical activity	Physical activity	<0.1 (0.1)	0.131	0.211
Internalizing behavior	Crude	Drinking alcohol in the past one year: yes	Intercept	1.5 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Drinking alcohol in the past one year: yes	Drinking alcohol in the past one year: yes	<0.1 (0.2)	0.402	0.517
Internalizing behavior	Crude	Smoking cigarettes in the past one year: yes	Intercept	1.4 (0.1)	<0.001	<0.001

Internalizing behavior	Crude	Smoking cigarettes in the past one year: yes	Smoking cigarettes in the past one year: yes	0.2 (0.4)	0.604	0.672
Internalizing behavior	Crude	Taking drugs in the past one year: yes	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Taking drugs in the past one year: yes	Taking drugs in the past one year: yes	0.2 (0.3)	0.56	0.64
Internalizing behavior	Crude	Bristol score	Intercept	1.4 (0.2)	<0.001	<0.001
Internalizing behavior	Crude	Bristol score	Bristol score	<0.1 (0.1)	0.892	0.935
Internalizing behavior	Crude	Maternal education level	Intercept	1.2 (0.3)	<0.001	<0.001
Internalizing behavior	Crude	Maternal education level	Maternal education level	<0.1 (<0.1)	0.355	0.486
Internalizing behavior	Crude	Paternal education level	Intercept	1.1 (0.2)	<0.001	<0.001
Internalizing behavior	Crude	Paternal education level	Paternal education level	0.1 (<0.1)	0.116	0.193
Internalizing behavior	Crude	Overnight sleep duration	Intercept	3.0 (0.4)	<0.001	<0.001
Internalizing behavior	***Crude***	***Overnight sleep duration***	***Overnight sleep duration***	***<0.1 (0.1)***	***<0.001***	***0.001***
Internalizing behavior	Crude	Pet: yes	Intercept	1.4 (0.1)	<0.001	<0.001
Internalizing behavior	Crude	Pet: yes	Pet: yes	0.1 (0.1)	0.506	0.607
Externalizing behavior	Crude	Age in years	Intercept	<0.1 (20.6)	0.865	0.916
Externalizing behavior	Crude	Age in years	Age in years	0.7 (1.4)	0.634	0.698
Externalizing behavior	Crude	Child gender: girl	Intercept	6.2 (0.3)	<0.001	<0.001
Externalizing behavior	Crude	Child gender: girl	Child gender: girl	0.4 (0.5)	0.467	0.573
Externalizing behavior	Crude	Thelarche or testicular development	Intercept	6.9 (1.2)	<0.001	<0.001
Externalizing behavior	Crude	Thelarche or testicular development	Thelarche or testicular development	<0.1 (0.3)	0.665	0.719
Externalizing behavior	Crude	Pubarche	Intercept	7.7 (1.1)	<0.001	<0.001
Externalizing behavior	Crude	Pubarche	Pubarche	<0.1 (0.3)	0.208	0.317
Externalizing behavior	Crude	zBMI	Intercept	6.3 (0.2)	<0.001	<0.001
Externalizing behavior	Crude	zBMI	zBMI	0.2 (0.2)	0.337	0.466
Externalizing behavior	Crude	Sick in the week before the home visit: yes	Intercept	6.4 (0.3)	<0.001	<0.001
Externalizing behavior	Crude	Sick in the week before the home visit: yes	Sick in the week before the home visit: yes	0.5 (1.2)	0.666	0.719
Externalizing behavior	Crude	Oral antibiotics in the past one year: yes	Intercept	6.4 (0.2)	<0.001	<0.001

Externalizing behavior	Crude	Oral antibiotics in the past one year: yes	Oral antibiotics in the past one year: yes	<0.1 (2.1)	0.171	0.268
Externalizing behavior	Crude	Diet quality	Intercept	7.7 (1.3)	<0.001	<0.001
Externalizing behavior	Crude	Diet quality	Diet quality	<0.1 (<0.1)	0.287	0.414
Externalizing behavior	Crude	Omega 3 fatty acids: yes	Intercept	6.4 (0.3)	<0.001	<0.001
Externalizing behavior	Crude	Omega 3 fatty acids: yes	Omega 3 fatty acids: yes	<0.1 (1.3)	0.446	0.554
Externalizing behavior	Crude	Physical activity	Intercept	6.2 (1.1)	<0.001	<0.001
Externalizing behavior	Crude	Physical activity	Physical activity	<0.1 (0.5)	0.918	0.944
Externalizing behavior	Crude	Drinking alcohol in the past one year: yes	Intercept	6.0 (0.3)	<0.001	<0.001
Externalizing behavior	***Crude***	***Drinking alcohol in the past one year: yes***	***Drinking alcohol in the past one year: yes***	***1.6 (0.6)***	***0.006***	***0.012***
Externalizing behavior	Crude	Smoking cigarettes in the past one year: yes	Intercept	6.3 (0.2)	<0.001	<0.001
Externalizing behavior	Crude	Smoking cigarettes in the past one year: yes	Smoking cigarettes in the past one year: yes	1.0 (1.5)	0.485	0.589
Externalizing behavior	Crude	Taking drugs in the past one year: yes	Intercept	6.3 (0.3)	<0.001	<0.001
Externalizing behavior	Crude	Taking drugs in the past one year: yes	Taking drugs in the past one year: yes	0.8 (1.3)	0.569	0.64
Externalizing behavior	Crude	Bristol score	Intercept	5.7 (0.9)	<0.001	<0.001
Externalizing behavior	Crude	Bristol score	Bristol score	0.2 (0.3)	0.561	0.64
Externalizing behavior	Crude	Maternal education level	Intercept	6.3 (1.1)	<0.001	<0.001
Externalizing behavior	Crude	Maternal education level	Maternal education level	<0.1 (0.2)	0.996	1
Externalizing behavior	Crude	Paternal education level	Intercept	6.3 (0.8)	<0.001	<0.001
Externalizing behavior	Crude	Paternal education level	Paternal education level	<0.1 (0.1)	0.992	1
Externalizing behavior	Crude	Overnight sleep duration	Intercept	12.7 (1.9)	<0.001	<0.001
Externalizing behavior	***Crude***	***Overnight sleep duration***	***Overnight sleep duration***	***<0.1 (0.2)***	***0.001***	***0.002***
Externalizing behavior	Crude	Pet: yes	Intercept	6.0 (0.4)	<0.001	<0.001
Externalizing behavior	Crude	Pet: yes	Pet: yes	0.5 (0.5)	0.374	0.492
Social anxiety	Crude	Age in years	Intercept	5.9 (2.2)	0.008	0.014
Social anxiety	Crude	Age in years	Age in years	<0.1 (0.1)	0.325	0.456
Social anxiety	Crude	Child gender: girl	Intercept	3.6 (<0.1)	<0.001	<0.001

Social anxiety	***Crude***	***Child gender: girl***	***Child gender: girl***	***0.2 (0.1)***	***0.003***	***0.005***
Social anxiety	Crude	Thelarche or testicular development	Intercept	3.9 (0.1)	<0.001	<0.001
Social anxiety	Crude	Thelarche or testicular development	Thelarche or testicular development	<0.1 (<0.1)	0.287	0.414
Social anxiety	Crude	Pubarche	Intercept	3.6 (0.1)	<0.001	<0.001
Social anxiety	Crude	Pubarche	Pubarche	<0.1 (<0.1)	0.443	0.554
Social anxiety	Crude	zBMI	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	zBMI	zBMI	<0.1 (<0.1)	0.915	0.944
Social anxiety	Crude	Sick in the week before the home visit: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Sick in the week before the home visit: yes	Sick in the week before the home visit: yes	<0.1 (0.1)	0.567	0.64
Social anxiety	Crude	Oral antibiotics in the past one year: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Oral antibiotics in the past one year: yes	Oral antibiotics in the past one year: yes	<0.1 (0.2)	0.09	0.155
Social anxiety	Crude	Diet quality	Intercept	3.4 (0.1)	<0.001	<0.001
Social anxiety	***Crude***	***Diet quality***	***Diet quality***	***<0.1 (<0.1)***	***0.026***	***0.047***
Social anxiety	Crude	Omega 3 fatty acids: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Omega 3 fatty acids: yes	Omega 3 fatty acids: yes	0.2 (0.1)	0.139	0.221
Social anxiety	Crude	Physical activity	Intercept	3.9 (0.1)	<0.001	<0.001
Social anxiety	Crude	Physical activity	Physical activity	<0.1 (<0.1)	0.112	0.19
Social anxiety	Crude	Drinking alcohol in the past one year: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Drinking alcohol in the past one year: yes	Drinking alcohol in the past one year: yes	<0.1 (0.1)	0.23	0.34
Social anxiety	Crude	Smoking cigarettes in the past one year: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Smoking cigarettes in the past one year: yes	Smoking cigarettes in the past one year: yes	0.1 (0.2)	0.525	0.616
Social anxiety	Crude	Taking drugs in the past one year: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Taking drugs in the past one year: yes	Taking drugs in the past one year: yes	0.1 (0.1)	0.372	0.492
Social anxiety	Crude	Bristol score	Intercept	3.6 (0.1)	<0.001	<0.001
Social anxiety	Crude	Bristol score	Bristol score	<0.1 (<0.1)	0.409	0.52
Social anxiety	Crude	Maternal education level	Intercept	3.5 (0.1)	<0.001	<0.001

Social anxiety	Crude	Maternal education level	Maternal education level	<0.1 (<0.1)	0.09	0.155
Social anxiety	Crude	Paternal education level	Intercept	3.5 (0.1)	<0.001	<0.001
Social anxiety	***Crude***	***Paternal education level***	***Paternal education level***	***<0.1 (<0.1)***	***0.008***	***0.016***
Social anxiety	Crude	Overnight sleep duration	Intercept	4.3 (0.2)	<0.001	<0.001
Social anxiety	***Crude***	***Overnight sleep duration***	***Overnight sleep duration***	***<0.1 (<0.1)***	***0.003***	***0.005***
Social anxiety	Crude	Pet: yes	Intercept	3.7 (<0.1)	<0.001	<0.001
Social anxiety	Crude	Pet: yes	Pet: yes	<0.1 (0.1)	1	1

Notes. Relations with original unadjusted p values < 0.05 are italicized and bold. Regarding Bristol stool consistency, we also treated it as a categorical variable consisting of two categories (i.e., Bristol scores <3 and ≥3), as only N = 5 children reported stools with Bristol scores higher than 4 as displayed in Figure S1. Similar to the numeric Bristol variable presented in the table, the categorical one was not significantly related to internalizing behavior, externalizing behavior, and social anxiety (unadjusted *p* values = 0.489, 0.385, and 0.779, respectively).

Table S2. Explained microbial variances at age 14 by potential covariates and behavioral measures.

	Variable	R^2 %	F	p-value (Pr>F)	Number of available samples
Simple effect(s)	Age in years	0.40	0.49	0.83	125
	Child gender (boy/girl)	1.47	1.84	0.10	125
	Thelarche or testicular development	0.69	0.85	0.49	125
	Pubarche	0.64	0.79	0.55	125
	zBMI	1.20	1.49	0.18	125
	Sick in the week before the home visit (yes/no)	0.50	0.62	0.66	124
	Oral antibiotics in the past one year (yes/no)	0.49	0.60	0.68	125
	Diet quality	0.99	1.17	0.29	119
	Omega-3 fatty acids (yes/no)	0.61	0.73	0.61	120
	Physical activity	0.42	0.50	0.84	119
	Drinking alcohol in the past one year (yes/no)	1.72	2.13	0.07	124
	Smoking cigarettes in the past one year (yes/no)	0.27	0.33	0.95	124
	Taking drugs in the past one year (yes/no)	0.27	0.33	0.93	124
	Bristol score (numeric)	0.78	0.93	0.45	120
	Bristol score (categories: Bristol scores <3 and ≥3)	0.79	0.94	0.44	
	Maternal education level	1.47	1.84	0.10	125
	Paternal education level	0.68	0.81	0.53	120
	Overnight sleep duration in hours	3.05	3.71	<0.01	120
	Pets (yes/no)	1.29	1.61	0.14	125
	Internalizing behavior	1.35	1.68	0.12	125
	Externalizing behavior	1.93	2.42	0.04	125
	Social anxiety	1.23	1.47	0.17	120

Marginal effect(s)	Overnight sleep duration in hours	2.07	2.52	0.03	119
	Drinking alcohol in the past one year (yes/no)	1.80	2.20	0.05	
	Externalizing behavior	0.58	0.71	0.60	

Notes. UniFrac distance was calculated on the genus-level relative abundance data prior to redundancy analysis. Regarding simple effects, redundancy analysis was performed on each variable separately, and therefore without considering any other variables. Marginal effects assess R^2% explained by one variable when R^2% explained by other variables was took out. To measure marginal effects, we selected variables with *p* values lower than 0.1 in simple effects and assessed them together in one model. In this case, overnight sleep duration in hours, drinking alcohol in the past one year (yes/no), and externalizing behavior were chosen accordingly. P values were obtained based on N = 999 permutation tests. We treated Bristol stool consistency data as either a numeric variable or a categorical variable consisting of two categories (Bristol scores <3 and ≥3), as N = 4 children reported a type between two known consecutive types (e.g., a type between type 3 and type 4) and only N = 5 children reported stools with Bristol scores higher than 4 as shown in Figure S1.

Table S3. Differences of potential covariates between pubertal clusters.

	Characteristic	Overall, N = 264[1]	Puberty_1, N = 74[1]	Puberty_2, N = 57[1]	Puberty_3, N = 46[1]	Puberty_4, N = 87[1]	p-value[2]	q-value[3]
12y and 14y	Age in years	13.5 (0.9)	13.6 (0.9)	13.5 (0.9)	13.6 (0.9)	13.4 (0.9)	0.4	0.6
	Child gender						0.009	0.2
	boy	142 / 264 (54%)	35 / 74 (47%)	38 / 57 (67%)	17 / 46 (37%)	52 / 87 (60%)		
	girl	122 / 264 (46%)	39 / 74 (53%)	19 / 57 (33%)	29 / 46 (63%)	35 / 87 (40%)		
	Thelarche or testicular development	3.3 (0.9)	3.5 (0.9)	3.3 (1.1)	3.3 (0.9)	3.3 (0.8)	0.5	0.6
	(Missing)	1	0	1	0	0		
	Pubarche	3.1 (1.1)	3.3 (1.1)	2.9 (1.1)	3.0 (1.1)	3.0 (1.0)	0.2	0.4
	(Missing)	1	0	1	0	0		
	BMI	19.1 (3.1)	19.4 (3.0)	19.7 (3.6)	19.2 (3.4)	18.4 (2.5)	0.061	0.3
	(Missing)	2	0	1	0	1		
	Oral antibiotics in the past one year (yes/overall)[4]	9 / 264 (3.4%)	6 / 74 (8.1%)	0 / 57 (0%)	2 / 46 (4.3%)	1 / 87 (1.1%)	0.034	0.2
	Characteristic	**Overall, N = 139[1]**	**Puberty_1, N = 36[1]**	**Puberty_2, N = 31[1]**	**Puberty_3, N = 22[1]**	**Puberty_4, N = 50[1]**	**p-value[2]**	**q-value[3]**
12y	Food factor 1[5]	0.0 (0.8)	-0.2 (0.7)	0.0 (1.0)	0.0 (0.8)	0.2 (0.8)	0.2	0.4
	(Missing)	2	0	1	0	1		
	Food factor 2[5]	0.0 (0.8)	-0.1 (0.9)	0.0 (0.8)	0.1 (0.7)	0.1 (0.8)	0.5	0.6
	(Missing)	2	0	1	0	1		

	Characteristic	Overall, N = 125[1]	Puberty_1, N = 38[1]	Puberty_2, N = 26[1]	Puberty_3, N = 24[1]	Puberty_4, N = 37[1]	p-value[2]	q-value[3]
14y	Diet quality[5]	87.0 (17.3)	83.5 (16.2)	86.6 (17.6)	90.9 (16.0)	88.5 (19.0)	0.5	0.6
	(Missing)	6	2	0	1	3		
	Omega-3 fatty acids (yes/overall)[6]	4 / 120 (3.3%)	1 / 36 (2.8%)	1 / 26 (3.8%)	0 / 24 (0%)	2 / 34 (5.9%)	0.8	0.8
	(Missing)	5	2	0	0	3		
	Physical activity	2.3 (0.5)	2.3 (0.5)	2.4 (0.5)	2.2 (0.5)	2.5 (0.5)	0.2	0.4
	(Missing)	6	3	0	0	3		
	Drinking alcohol in the past one year (yes/overall)	22 / 124 (18%)	5 / 37 (14%)	10 / 26 (38%)	1 / 24 (4.2%)	6 / 37 (16%)	0.016	0.2
	(Missing)	1	1	0	0	0		
	Smoking cigarettes in the past one year (yes/overall)	3 / 124 (2.4%)	0 / 37 (0%)	0 / 26 (0%)	1 / 24 (4.2%)	2 / 37 (5.4%)	0.4	0.6
	(Missing)	1	1	0	0	0		
	Taking drugs in the past one year (yes/overall)	4 / 124 (3.2%)	0 / 37 (0%)	0 / 26 (0%)	1 / 24 (4.2%)	3 / 37 (8.1%)	0.2	0.4
	(Missing)	1	1	0	0	0		
	Bristol score	3.1 (1.0)	3.0 (1.2)	3.0 (0.9)	3.0 (0.9)	3.4 (0.7)	0.1	0.4
	(Missing)	5	1	0	1	3		
	Maternal education level	5.9 (1.3)	5.6 (1.5)	6.0 (1.4)	6.0 (1.2)	6.0 (1.3)	0.6	0.6
	Paternal education level	5.4 (1.8)	5.4 (1.8)	5.0 (2.1)	5.2 (1.8)	5.7 (1.7)	0.6	0.6
	(Missing)	5	1	1	3	0		
	Overnight sleep duration in hours	8.2 (1.1)	8.3 (0.9)	7.8 (1.4)	8.3 (0.9)	8.5 (1.0)	0.2	0.4
	(Missing)	5	2	0	0	3		
	Pets	82 / 125 (66%)	23 / 38 (61%)	21 / 26 (81%)	16 / 24 (67%)	22 / 37 (59%)	0.3	0.5

[1]Mean (SD); n / N (%)

[2]Kruskal-Wallis rank sum test; Pearson's Chi-squared test; Fisher's exact test

[3]False discovery rate correction for multiple testing

[4]Non-oral antibiotics were excluded

[5]Diet was compared separately for clusters at the age of 12 and 14 years, as different food questionnaires were used. Two food factors were obtained from one of our earlier studies, with factor 1 representing healthy foods and factor 2 referring to snacks.
[6]Another diet relevant variable, i.e., probiotic consumption, is not shown, as no children took probiotics at age 14.

Table S4. Relations between microbial clusters in the first 14 years of life and behavioral outcomes at age 14.

Behavior	Age	Model	Parameter	Estimate (Std. Error)	p	Adjusted p	VIF
Internalizing behavior	1m	Crude	Intercept	1.3 (0.1)	<0.001	<0.001	NA
Internalizing behavior	1m	Crude	Clusters: Infancy_2	0.1 (0.2)	0.597	0.749	NA
Internalizing behavior	1m	Crude	Clusters: Infancy_3	<0.1 (0.2)	0.965	0.976	NA
Internalizing behavior	3m	Crude	Intercept	1.3 (0.1)	<0.001	<0.001	NA
Internalizing behavior	3m	Crude	Clusters: Infancy_2	0.2 (0.1)	0.144	0.259	NA
Internalizing behavior	3m	Crude	Clusters: Infancy_3	<0.1 (0.2)	0.735	0.83	NA
Internalizing behavior	4m	Crude	Intercept	1.4 (0.1)	<0.001	<0.001	NA
Internalizing behavior	4m	Crude	Clusters: Infancy_2	0.1 (0.1)	0.393	0.566	NA
Internalizing behavior	4m	Crude	Clusters: Infancy_3	<0.1 (0.2)	0.809	0.893	NA
Internalizing behavior	6y	Crude	Intercept	1.5 (0.1)	<0.001	<0.001	NA
Internalizing behavior	6y	Crude	Clusters: Childhood_2	0.1 (0.2)	0.71	0.829	NA
Internalizing behavior	6y	Crude	Clusters: Childhood_3	<0.1 (0.2)	0.055	0.118	NA
Internalizing behavior	6y	Crude	Clusters: Childhood_4	<0.1 (0.2)	0.974	0.979	NA
Internalizing behavior	10y	Crude	Intercept	1.5 (0.1)	<0.001	<0.001	NA
Internalizing behavior	10y	Crude	Clusters: Childhood_2	<0.1 (0.2)	0.812	0.893	NA
Internalizing behavior	10y	Crude	Clusters: Childhood_3	<0.1 (0.2)	0.495	0.658	NA
Internalizing behavior	10y	Crude	Clusters: Childhood_4	<0.1 (0.2)	0.614	0.749	NA
Internalizing behavior	12y	Crude	Intercept	1.6 (0.1)	<0.001	<0.001	NA
Internalizing behavior	12y	Crude	Clusters: Puberty_2	<0.1 (0.2)	0.468	0.651	NA
Internalizing behavior	12y	Crude	Clusters: Puberty_3	0.1 (0.2)	0.726	0.83	NA
Internalizing behavior	12y	Crude	Clusters: Puberty_4	<0.1 (0.2)	0.157	0.269	NA
Internalizing behavior	14y	Crude	Intercept	1.4 (0.1)	<0.001	<0.001	NA
Internalizing behavior	14y	Crude	Clusters: Puberty_2	0.3 (0.2)	0.131	0.245	NA

Internalizing behavior	14y	Crude	Clusters: Puberty_3	0.1 (0.2)	0.495	0.658	NA
Internalizing behavior	14y	Crude	Clusters: Puberty_4	<0.1 (0.2)	0.291	0.437	NA
Internalizing behavior	Infancy	Crude	Intercept	1.3 (0.1)	<0.001	<0.001	NA
Internalizing behavior	Infancy	Crude	Clusters: Infancy_2	0.2 (0.1)	0.044	0.102	NA
Internalizing behavior	Infancy	Crude	Clusters: Infancy_3	<0.1 (0.1)	0.735	0.83	NA
Internalizing behavior	Childhood	Crude	Intercept	1.5 (0.1)	<0.001	<0.001	NA
Internalizing behavior	Childhood	Crude	Clusters: Childhood_2	<0.1 (0.1)	0.874	0.942	NA
Internalizing behavior	Childhood	Crude	Clusters: Childhood_3	<0.1 (0.1)	0.103	0.202	NA
Internalizing behavior	Childhood	Crude	Clusters: Childhood_4	<0.1 (0.1)	0.716	0.83	NA
Internalizing behavior	Puberty	Crude	Intercept	1.5 (0.1)	<0.001	<0.001	NA
Internalizing behavior	Puberty	Crude	Clusters: Puberty_2	0.1 (0.1)	0.586	0.742	NA
Internalizing behavior	Puberty	Crude	Clusters: Puberty_3	0.1 (0.1)	0.473	0.653	NA
Internalizing behavior	Puberty	Crude	Clusters: Puberty_4	<0.1 (0.1)	0.099	0.199	NA
Externalizing behavior	1m	Crude	Intercept	6.3 (0.3)	<0.001	<0.001	NA
Externalizing behavior	1m	Crude	Clusters: Infancy_2	0.5 (0.9)	0.608	0.749	NA
Externalizing behavior	1m	Crude	Clusters: Infancy_3	<0.1 (0.7)	0.703	0.827	NA
Externalizing behavior	3m	Crude	Intercept	6.4 (0.4)	<0.001	<0.001	NA
Externalizing behavior	3m	Crude	Clusters: Infancy_2	<0.1 (0.6)	0.997	0.997	NA
Externalizing behavior	3m	Crude	Clusters: Infancy_3	<0.1 (0.9)	0.386	0.561	NA
Externalizing behavior	4m	Crude	Intercept	6.0 (0.4)	<0.001	<0.001	NA
Externalizing behavior	4m	Crude	Clusters: Infancy_2	0.9 (0.6)	0.126	0.243	NA
Externalizing behavior	4m	Crude	Clusters: Infancy_3	<0.1 (0.9)	0.281	0.427	NA
Externalizing behavior	6y	Crude	Intercept	5.4 (0.4)	<0.001	<0.001	NA
Externalizing behavior	6y	Crude	Clusters: Childhood_2	1.9 (0.7)	0.008	0.023	NA
Externalizing behavior	6y	Crude	Clusters: Childhood_3	0.9 (0.7)	0.184	0.301	NA
Externalizing behavior	6y	Crude	Clusters: Childhood_4	0.6 (0.7)	0.382	0.56	NA

Externalizing behavior	10y	Crude	Intercept	5.6 (0.5)	<0.001	<0.001	NA
Externalizing behavior	10y	Crude	Clusters: Childhood_2	1.4 (0.9)	0.136	0.248	NA
Externalizing behavior	10y	Crude	Clusters: Childhood_3	0.5 (0.7)	0.481	0.658	NA
Externalizing behavior	10y	Crude	Clusters: Childhood_4	0.7 (0.7)	0.312	0.466	NA
Externalizing behavior	12y	Crude	Intercept	6.0 (0.5)	<0.001	<0.001	NA
Externalizing behavior	12y	Crude	Clusters: Puberty_2	1.9 (0.7)	0.012	0.033	NA
Externalizing behavior	12y	Crude	Clusters: Puberty_3	1.0 (0.8)	0.219	0.348	NA
Externalizing behavior	12y	Crude	Clusters: Puberty_4	<0.1 (0.7)	0.427	0.61	NA
Externalizing behavior	14y	Crude	Intercept	5.8 (0.5)	<0.001	<0.001	NA
Externalizing behavior	14y	Crude	Clusters: Puberty_2	1.8 (0.7)	0.012	0.034	NA
Externalizing behavior	14y	Crude	Clusters: Puberty_3	0.5 (0.7)	0.497	0.658	NA
Externalizing behavior	14y	Crude	Clusters: Puberty_4	<0.1 (0.7)	0.557	0.71	NA
Externalizing behavior	Infancy	Crude	Intercept	6.3 (0.2)	<0.001	<0.001	NA
Externalizing behavior	Infancy	Crude	Clusters: Infancy_2	0.4 (0.4)	0.222	0.35	NA
Externalizing behavior	Infancy	Crude	Clusters: Infancy_3	<0.1 (0.5)	0.204	0.327	NA
Externalizing behavior	Childhood	Crude	Intercept	5.5 (0.3)	<0.001	<0.001	NA
Externalizing behavior	Childhood	Crude	Clusters: Childhood_2	1.7 (0.5)	0.002	0.009	NA
Externalizing behavior	Childhood	Crude	Clusters: Childhood_3	0.7 (0.5)	0.136	0.248	NA
Externalizing behavior	Childhood	Crude	Clusters: Childhood_4	0.7 (0.5)	0.13	0.245	NA
Externalizing behavior	Puberty	Crude	Intercept	5.9 (0.3)	<0.001	<0.001	NA
Externalizing behavior	Puberty	Crude	Clusters: Puberty_2	1.9 (0.5)	<0.001	0.001	NA
Externalizing behavior	Puberty	Crude	Clusters: Puberty_3	0.7 (0.5)	0.176	0.291	NA
Externalizing behavior	Puberty	Crude	Clusters: Puberty_4	<0.1 (0.5)	0.331	0.489	NA
Social anxiety	1m	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	1m	Crude	Clusters: Infancy_2	0.1 (0.1)	0.53	0.692	NA
Social anxiety	1m	Crude	Clusters: Infancy_3	<0.1 (0.1)	0.908	0.96	NA
Social anxiety	3m	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	3m	Crude	Clusters: Infancy_2	0.1 (0.1)	0.033	0.078	NA
Social anxiety	3m	Crude	Clusters: Infancy_3	<0.1 (0.1)	0.813	0.893	NA

Social anxiety	4m	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	4m	Crude	Clusters: Infancy_2	<0.1 (0.1)	0.754	0.846	NA
Social anxiety	4m	Crude	Clusters: Infancy_3	<0.1 (0.1)	0.922	0.968	NA
Social anxiety	6y	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	6y	Crude	Clusters: Childhood_2	<0.1 (0.1)	0.904	0.96	NA
Social anxiety	6y	Crude	Clusters: Childhood_3	<0.1 (0.1)	0.152	0.264	NA
Social anxiety	6y	Crude	Clusters: Childhood_4	<0.1 (0.1)	0.824	0.899	NA
Social anxiety	10y	Crude	Intercept	3.7 (0.1)	<0.001	<0.001	NA
Social anxiety	10y	Crude	Clusters: Childhood_2	<0.1 (0.1)	0.957	0.974	NA
Social anxiety	10y	Crude	Clusters: Childhood_3	<0.1 (0.1)	0.55	0.706	NA
Social anxiety	10y	Crude	Clusters: Childhood_4	<0.1 (0.1)	0.908	0.96	NA
Social anxiety	12y	Crude	Intercept	3.7 (0.1)	<0.001	<0.001	NA
Social anxiety	12y	Crude	Clusters: Puberty_2	<0.1 (0.1)	0.609	0.749	NA
Social anxiety	12y	Crude	Clusters: Puberty_3	<0.1 (0.1)	0.661	0.783	NA
Social anxiety	12y	Crude	Clusters: Puberty_4	<0.1 (0.1)	0.65	0.775	NA
Social anxiety	14y	Crude	Intercept	3.6 (<0.1)	<0.001	<0.001	NA
Social anxiety	14y	Crude	Clusters: Puberty_2	0.1 (0.1)	0.101	0.201	NA
Social anxiety	14y	Crude	Clusters: Puberty_3	0.2 (0.1)	0.017	0.044	NA
Social anxiety	14y	Crude	Clusters: Puberty_4	<0.1 (0.1)	0.633	0.76	NA
Social anxiety	Infancy	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	Infancy	Crude	Clusters: Infancy_2	0.1 (<0.1)	0.058	0.123	NA
Social anxiety	Infancy	Crude	Clusters: Infancy_3	<0.1 (0.1)	0.873	0.942	NA
Social anxiety	Childhood	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	Childhood	Crude	Clusters: Childhood_2	<0.1 (0.1)	0.951	0.974	NA
Social anxiety	Childhood	Crude	Clusters: Childhood_3	<0.1 (0.1)	0.16	0.269	NA
Social anxiety	Childhood	Crude	Clusters: Childhood_4	<0.1 (0.1)	0.951	0.974	NA
Social anxiety	Puberty	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	Puberty	Crude	Clusters: Puberty_2	<0.1 (0.1)	0.449	0.63	NA
Social anxiety	Puberty	Crude	Clusters: Puberty_3	0.1 (0.1)	0.048	0.107	NA
Social anxiety	Puberty	Crude	Clusters: Puberty_4	<0.1 (<0.1)	0.933	0.972	NA
Internalizing behavior	Infancy	Adjusted	Intercept	1.6 (0.3)	<0.001	<0.001	NA
Internalizing behavior	Infancy	Adjusted	Clusters: Infancy_2	0.2 (0.1)	0.007	0.021	1.02
Internalizing behavior	Infancy	Adjusted	Clusters: Infancy_3	<0.1 (0.1)	0.937	0.972	1.02
Internalizing behavior	Infancy	Adjusted	Child gender: girl	0.4 (0.1)	<0.001	<0.001	1.05
Internalizing behavior	Infancy	Adjusted	Diet quality	<0.1 (<0.1)	0.001	0.002	1.07

Internalizing behavior	Infancy	Adjusted	Overnight sleep duration	<0.1 (<0.1)	<0.001	<0.001	1.03
Externalizing behavior	6y	Adjusted	Intercept	10.0 (2.0)	<0.001	<0.001	NA
Externalizing behavior	6y	Adjusted	Clusters: Childhood_2	1.6 (0.7)	0.025	0.06	1.13
Externalizing behavior	6y	Adjusted	Clusters: Childhood_3	1.1 (0.7)	0.094	0.192	1.13
Externalizing behavior	6y	Adjusted	Clusters: Childhood_4	0.8 (0.7)	0.261	0.4	1.13
Externalizing behavior	6y	Adjusted	Drinking alcohol in the past one year: yes	1.4 (0.7)	0.04	0.094	1.05
Externalizing behavior	6y	Adjusted	Overnight sleep duration	<0.1 (0.2)	0.017	0.044	1.09
Externalizing behavior	12y	Adjusted	Intercept	10.8 (2.0)	<0.001	<0.001	NA
Externalizing behavior	12y	Adjusted	Clusters: Puberty_2	1.7 (0.7)	0.021	0.054	1.06
Externalizing behavior	12y	Adjusted	Clusters: Puberty_3	1.2 (0.8)	0.15	0.264	1.06
Externalizing behavior	12y	Adjusted	Clusters: Puberty_4	<0.1 (0.7)	0.725	0.83	1.06
Externalizing behavior	12y	Adjusted	Drinking alcohol in the past one year: yes	1.5 (0.6)	0.023	0.057	1.03
Externalizing behavior	12y	Adjusted	Overnight sleep duration	<0.1 (0.2)	0.01	0.028	1.04
Externalizing behavior	14y	Adjusted	Intercept	10.9 (2.1)	<0.001	<0.001	NA
Externalizing behavior	14y	Adjusted	Clusters: Puberty_2	1.0 (0.7)	0.19	0.308	1.18
Externalizing behavior	14y	Adjusted	Clusters: Puberty_3	0.4 (0.7)	0.627	0.758	1.18
Externalizing behavior	14y	Adjusted	Clusters: Puberty_4	<0.1 (0.7)	0.49	0.658	1.18
Externalizing behavior	14y	Adjusted	Drinking alcohol in the past one year: yes	1.1 (0.7)	0.137	0.248	1.13
Externalizing behavior	14y	Adjusted	Overnight sleep duration	<0.1 (0.2)	0.017	0.044	1.06
Externalizing behavior	Childhood	Adjusted	Intercept	10.5 (1.4)	<0.001	<0.001	NA
Externalizing behavior	Childhood	Adjusted	Clusters: Childhood_2	1.4 (0.5)	0.008	0.023	1.07
Externalizing behavior	Childhood	Adjusted	Clusters: Childhood_3	1.1 (0.5)	0.023	0.056	1.07
Externalizing behavior	Childhood	Adjusted	Clusters: Childhood_4	0.9 (0.5)	0.047	0.105	1.07
Externalizing behavior	Childhood	Adjusted	Drinking alcohol in the past one year: yes	1.6 (0.5)	0.001	0.003	1.03
Externalizing behavior	Childhood	Adjusted	Overnight sleep duration	<0.1 (0.2)	<0.001	<0.001	1.05
Externalizing behavior	Puberty	Adjusted	Intercept	10.8 (1.4)	<0.001	<0.001	NA

Externalizing behavior	Puberty	Adjusted	Clusters: Puberty_2	1.3 (0.5)	0.01	0.029	1.08
Externalizing behavior	Puberty	Adjusted	Clusters: Puberty_3	0.7 (0.5)	0.17	0.284	1.08
Externalizing behavior	Puberty	Adjusted	Clusters: Puberty_4	<0.1 (0.5)	0.434	0.615	1.08
Externalizing behavior	Puberty	Adjusted	Drinking alcohol in the past one year: yes	1.3 (0.5)	0.006	0.019	1.06
Externalizing behavior	Puberty	Adjusted	Overnight sleep duration	<0.1 (0.2)	<0.001	0.002	1.04
Social anxiety	3m	Adjusted	Intercept	3.4 (0.3)	<0.001	<0.001	NA
Social anxiety	3m	Adjusted	Clusters: Infancy_2	0.2 (0.1)	0.003	0.012	1.08
Social anxiety	3m	Adjusted	Clusters: Infancy_3	<0.1 (0.1)	0.61	0.749	1.08
Social anxiety	3m	Adjusted	Child gender: girl	0.1 (0.1)	0.147	0.262	1.05
Social anxiety	3m	Adjusted	Diet quality	<0.1 (<0.1)	0.082	0.168	1.26
Social anxiety	3m	Adjusted	Overnight sleep duration	<0.1 (<0.1)	0.259	0.4	1.05
Social anxiety	3m	Adjusted	Paternal education level	<0.1 (<0.1)	0.126	0.243	1.16
Social anxiety	14y	Adjusted	Intercept	3.5 (0.3)	<0.001	<0.001	NA
Social anxiety	14y	Adjusted	Clusters: Puberty_2	0.2 (0.1)	0.04	0.093	1.17
Social anxiety	14y	Adjusted	Clusters: Puberty_3	0.2 (0.1)	0.015	0.04	1.17
Social anxiety	14y	Adjusted	Clusters: Puberty_4	<0.1 (0.1)	0.549	0.706	1.17
Social anxiety	14y	Adjusted	Child gender: girl	0.1 (0.1)	0.05	0.11	1.06
Social anxiety	14y	Adjusted	Diet quality	<0.1 (<0.1)	0.522	0.686	1.2
Social anxiety	14y	Adjusted	Overnight sleep duration	<0.1 (<0.1)	0.249	0.389	1.15
Social anxiety	14y	Adjusted	Paternal education level	<0.1 (<0.1)	0.007	0.021	1.17
Social anxiety	Puberty	Adjusted	Intercept	3.6 (0.2)	<0.001	<0.001	NA
Social anxiety	Puberty	Adjusted	Clusters: Puberty_2	0.1 (0.1)	0.074	0.154	1.11
Social anxiety	Puberty	Adjusted	Clusters: Puberty_3	0.2 (0.1)	0.004	0.012	1.11
Social anxiety	Puberty	Adjusted	Clusters: Puberty_4	0.1 (<0.1)	0.159	0.269	1.11
Social anxiety	Puberty	Adjusted	Child gender: girl	0.1 (<0.1)	0.016	0.042	1.08
Social anxiety	Puberty	Adjusted	Diet quality	<0.1 (<0.1)	0.069	0.145	1.18

Social anxiety	Puberty	Adjusted	Overnight sleep duration	<0.1 (<0.1)	0.005	0.017	1.09
Social anxiety	Puberty	Adjusted	Paternal education level	<0.1 (<0.1)	0.003	0.011	1.14

Notes. Adjusted models were implemented only when microbial predictor estimates had original unadjusted $p < 0.05$ in crude models. In case of multicollinearity, VIF values were measured in adjusted models, and hereby not applicable (NA) for crude models.

Table S5. Relations between phylogenetic diversity in the first 14 years of life and behavioral outcomes at age 14.

Behavior	Age	Model	Parameter	Estimate (Std. Error)	p	Adjusted p	VIF
Internalizing behavior	1m	Crude	Intercept	1.1 (0.2)	<0.001	<0.001	NA
Internalizing behavior	1m	Crude	Phylogenetic diversity	0.1 (0.1)	0.346	0.531	NA
Internalizing behavior	3m	Crude	Intercept	1.1 (0.2)	<0.001	<0.001	NA
Internalizing behavior	3m	Crude	Phylogenetic diversity	0.1 (<0.1)	0.139	0.224	NA
Internalizing behavior	4m	Crude	Intercept	1.3 (0.2)	<0.001	<0.001	NA
Internalizing behavior	4m	Crude	Phylogenetic diversity	<0.1 (0.1)	0.551	0.712	NA
Internalizing behavior	6y	Crude	Intercept	1.7 (0.5)	0.001	0.001	NA
Internalizing behavior	6y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.57	0.715	NA
Internalizing behavior	10y	Crude	Intercept	1.7 (0.4)	<0.001	<0.001	NA
Internalizing behavior	10y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.439	0.631	NA
Internalizing behavior	12y	Crude	Intercept	1.5 (0.4)	<0.001	<0.001	NA
Internalizing behavior	12y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.989	0.989	NA
Internalizing behavior	14y	Crude	Intercept	1.5 (0.4)	<0.001	<0.001	NA
Internalizing behavior	14y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.737	0.869	NA
Internalizing behavior	Infancy	Crude	Intercept	1.2 (0.1)	<0.001	<0.001	NA
Internalizing behavior	Infancy	Crude	Phylogenetic diversity	0.1 (<0.1)	0.099	0.163	NA
Internalizing behavior	Childhood	Crude	Intercept	1.7 (0.3)	<0.001	<0.001	NA
Internalizing behavior	Childhood	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.355	0.533	NA
Internalizing behavior	Puberty	Crude	Intercept	1.5 (0.3)	<0.001	<0.001	NA
Internalizing behavior	Puberty	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.806	0.902	NA
Externalizing behavior	1m	Crude	Intercept	6.3 (0.8)	<0.001	<0.001	NA
Externalizing behavior	1m	Crude	Phylogenetic diversity	<0.1 (0.2)	0.924	0.953	NA
Externalizing behavior	3m	Crude	Intercept	6.5 (0.8)	<0.001	<0.001	NA

Externalizing behavior	3m	Crude	Phylogenetic diversity	<0.1 (0.2)	0.794	0.902	NA
Externalizing behavior	4m	Crude	Intercept	6.0 (0.9)	<0.001	<0.001	NA
Externalizing behavior	4m	Crude	Phylogenetic diversity	0.1 (0.3)	0.764	0.885	NA
Externalizing behavior	6y	Crude	Intercept	5.8 (1.8)	0.002	0.003	NA
Externalizing behavior	6y	Crude	Phylogenetic diversity	<0.1 (0.2)	0.866	0.937	NA
Externalizing behavior	10y	Crude	Intercept	5.1 (1.6)	0.002	0.003	NA
Externalizing behavior	10y	Crude	Phylogenetic diversity	0.1 (0.1)	0.467	0.656	NA
Externalizing behavior	12y	Crude	Intercept	5.8 (1.6)	<0.001	0.001	NA
Externalizing behavior	12y	Crude	Phylogenetic diversity	<0.1 (0.1)	0.701	0.842	NA
Externalizing behavior	14y	Crude	Intercept	5.4 (1.6)	0.001	0.003	NA
Externalizing behavior	14y	Crude	Phylogenetic diversity	0.1 (0.1)	0.642	0.785	NA
Externalizing behavior	Infancy	Crude	Intercept	6.3 (0.5)	<0.001	<0.001	NA
Externalizing behavior	Infancy	Crude	Phylogenetic diversity	<0.1 (0.1)	0.938	0.953	NA
Externalizing behavior	Childhood	Crude	Intercept	5.4 (1.2)	<0.001	<0.001	NA
Externalizing behavior	Childhood	Crude	Phylogenetic diversity	0.1 (0.1)	0.488	0.671	NA
Externalizing behavior	Puberty	Crude	Intercept	5.6 (1.1)	<0.001	<0.001	NA
Externalizing behavior	Puberty	Crude	Phylogenetic diversity	0.1 (0.1)	0.55	0.712	NA
Social anxiety	1m	Crude	Intercept	3.6 (0.1)	<0.001	<0.001	NA
Social anxiety	1m	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.521	0.701	NA
Social anxiety	3m	Crude	Intercept	3.5 (0.1)	<0.001	<0.001	NA
Social anxiety	3m	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.065	0.11	NA
Social anxiety	4m	Crude	Intercept	3.5 (0.1)	<0.001	<0.001	NA
Social anxiety	4m	Crude	Phylogenetic diversity	0.1 (<0.1)	0.057	0.099	NA
Social anxiety	6y	Crude	Intercept	3.7 (0.2)	<0.001	<0.001	NA
Social anxiety	6y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.885	0.942	NA
Social anxiety	10y	Crude	Intercept	3.7 (0.2)	<0.001	<0.001	NA
Social anxiety	10y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.854	0.937	NA
Social anxiety	12y	Crude	Intercept	3.6 (0.2)	<0.001	<0.001	NA
Social anxiety	12y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.404	0.592	NA

Social anxiety	14y	Crude	Intercept	3.6 (0.2)	<0.001	<0.001	NA
Social anxiety	14y	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.575	0.715	NA
Social anxiety	Infancy	Crude	Intercept	3.6 (0.1)	<0.001	<0.001	NA
Social anxiety	Infancy	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.016	0.03	NA
Social anxiety	Childhood	Crude	Intercept	3.7 (0.1)	<0.001	<0.001	NA
Social anxiety	Childhood	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.922	0.953	NA
Social anxiety	Puberty	Crude	Intercept	3.6 (0.1)	<0.001	<0.001	NA
Social anxiety	Puberty	Crude	Phylogenetic diversity	<0.1 (<0.1)	0.316	0.496	NA
Social anxiety	Infancy	Adjusted	Intercept	3.5 (0.2)	<0.001	<0.001	NA
Social anxiety	Infancy	Adjusted	Phylogenetic diversity	<0.1 (<0.1)	0.002	0.004	1.03
Social anxiety	Infancy	Adjusted	Child gender: girl	0.1 (<0.1)	0.018	0.034	1.05
Social anxiety	Infancy	Adjusted	Diet quality	<0.1 (<0.1)	0.03	0.053	1.25
Social anxiety	Infancy	Adjusted	Overnight sleep duration	<0.1 (<0.1)	0.003	0.006	1.05
Social anxiety	Infancy	Adjusted	Paternal education level	<0.1 (<0.1)	<0.001	0.001	1.16

Notes. Adjusted models were implemented only when microbial predictor estimates had original unadjusted $p < 0.05$ in crude models. In case of multicollinearity, VIF values were measured in adjusted models, and hereby not applicable (NA) for crude models.

Table S6. Differences in behavioral relations between disperse Puberty_2 samples and other samples in puberty.

Behavior	Age	Model	Parameter	Estimate (Std. Error)	p	Adjusted p	VIF
Internalizing behavior	12y	Crude	Intercept	1.5 (0.1)	<0.001	<0.001	NA
Internalizing behavior	12y	Crude	Group: disperse samples in Puberty_2	<0.1 (0.2)	0.699	0.726	NA
Internalizing behavior	14y	Crude	Intercept	1.3 (0.1)	<0.001	<0.001	NA
Internalizing behavior	14y	Crude	Group: disperse samples in Puberty_2	0.4 (0.2)	0.011	0.02	NA
Internalizing behavior	Puberty	Crude	Intercept	1.4 (<0.1)	<0.001	<0.001	NA
Internalizing behavior	Puberty	Crude	Group: disperse samples in Puberty_2	0.2 (0.1)	0.106	0.136	NA
Externalizing behavior	12y	Crude	Intercept	6.2 (0.3)	<0.001	<0.001	NA
Externalizing behavior	12y	Crude	Group: disperse samples in Puberty_2	1.1 (0.9)	0.214	0.251	NA
Externalizing behavior	14y	Crude	Intercept	6.0 (0.3)	<0.001	<0.001	NA
Externalizing behavior	14y	Crude	Group: disperse samples in Puberty_2	1.5 (0.7)	0.054	0.079	NA
Externalizing behavior	Puberty	Crude	Intercept	6.1 (0.2)	<0.001	<0.001	NA
Externalizing behavior	Puberty	Crude	Group: disperse samples in Puberty_2	1.3 (0.6)	0.026	0.041	NA
Social anxiety	12y	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	12y	Crude	Group: disperse samples in Puberty_2	<0.1 (0.1)	0.936	0.936	NA
Social anxiety	14y	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	14y	Crude	Group: disperse samples in Puberty_2	0.1 (0.1)	0.059	0.079	NA
Social anxiety	Puberty	Crude	Intercept	3.7 (<0.1)	<0.001	<0.001	NA
Social anxiety	Puberty	Crude	Group: disperse samples in Puberty_2	0.1 (0.1)	0.185	0.227	NA
Internalizing behavior	14y	Adjusted	Intercept	1.9 (0.5)	<0.001	0.001	NA
Internalizing behavior	14y	Adjusted	Group: disperse samples in Puberty_2	0.3 (0.2)	0.057	0.079	1.11
Internalizing behavior	14y	Adjusted	Child gender: girl	0.5 (0.1)	<0.001	<0.001	1.05
Internalizing behavior	14y	Adjusted	Diet quality	<0.1 (<0.1)	0.369	0.399	1.08
Internalizing behavior	14y	Adjusted	Overnight sleep duration	<0.1 (0.1)	0.02	0.034	1.12
Externalizing behavior	Puberty	Adjusted	Intercept	11.4 (1.5)	<0.001	<0.001	NA

Externalizing behavior	Puberty	Adjusted	Group: disperse samples in Puberty_2	0.6 (0.6)	0.3	0.338	1.05
Externalizing behavior	Puberty	Adjusted	Drinking alcohol in the past one year: yes	1.6 (0.5)	0.001	0.002	1.02
Externalizing behavior	Puberty	Adjusted	Overnight sleep duration	<0.1 (0.2)	<0.001	<0.001	1.05

Notes. A positive estimate for parameter group indicates higher behavioral scores in disperse Puberty_2 samples, in comparison with other samples in puberty. Adjusted models were implemented only when microbial predictor estimates had original unadjusted $p < 0.05$ in crude models. In case of multicollinearity, VIF values were measured in adjusted models, and hereby not applicable (NA) for crude models.

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Chapter 6

General discussion and future perspectives

In the research described in this thesis, we (1) described gut microbiota development in low-risk community children from birth to the age of 14 years, along with microbiota-related factors, and (2) explored relations between the gut microbiota and mental health in aspects of problem behavior, executive functions, prosociality, and social anxiety, in the same children. In the following sections, I will discuss our keys findings, including enduring development of the gut microbiota, the extended role of early-life breastfeeding in relation to microbial composition, and the microbiota-behavior interplay in low-risk children. This discussion will be followed by addressing necessary steps on the path from correlation to causation, the selection of confounders, and the use of integrative analytical approaches.

Gut microbiota development is a long dynamic journey

It is widely believed that child gut microbiota reaches a status of maturity, which highly resembles adult microbial profiles, within the first three years of life (Yatsunenko et al., 2012). However, according to the findings reported in this thesis (**Chapter 3** and **Chapter 5**) and earlier studies (Agans et al., 2011; Cheng et al., 2016; Hollister et al., 2015; Ringel-Kulka et al., 2013), a stable maturation stage may arrive later than previously expected, probably as a result of numerous extrinsic factors.

Herein, we identified three gut microbial clusters in infancy, four in middle childhood, and another four in puberty, which exhibited different compositional features. Remarkably, infant clusters conformed to *Bifidobacterium*-enriched and -depleted groups which were reported earlier (Borewicz et al., 2019; Dogra et al., 2015; Hill et al., 2017; Matsuki et al., 2016; Roswall et al., 2021; Stewart et al., 2018).

Based on Figure 1 in **Chapter 3** and Figure 1a in **Chapter 5**, we observed four potentially predominant transitional patterns (TPs; characterized as displaying a transitional rate higher than 10% between every two consecutive microbial clusters) that proceed from the clusters determined in middle childhood (ages of six and ten years) to those observed in puberty (ages of 12 and 14 years). In addition to these main TPs (TP1, TP2, TP4, and TP5; with the numberings determined by the order of clusters; Figure 1a), we found a fifth TP (TP3) predominated by *Prevotella* 9, although TP3 did not exceed the 10% transitional rate. TP3 included transitions between Childhood_2 and Puberty_2 in this period. Whether these predominant TPs exist in another cohort or different populations awaits to be confirmed in the future.

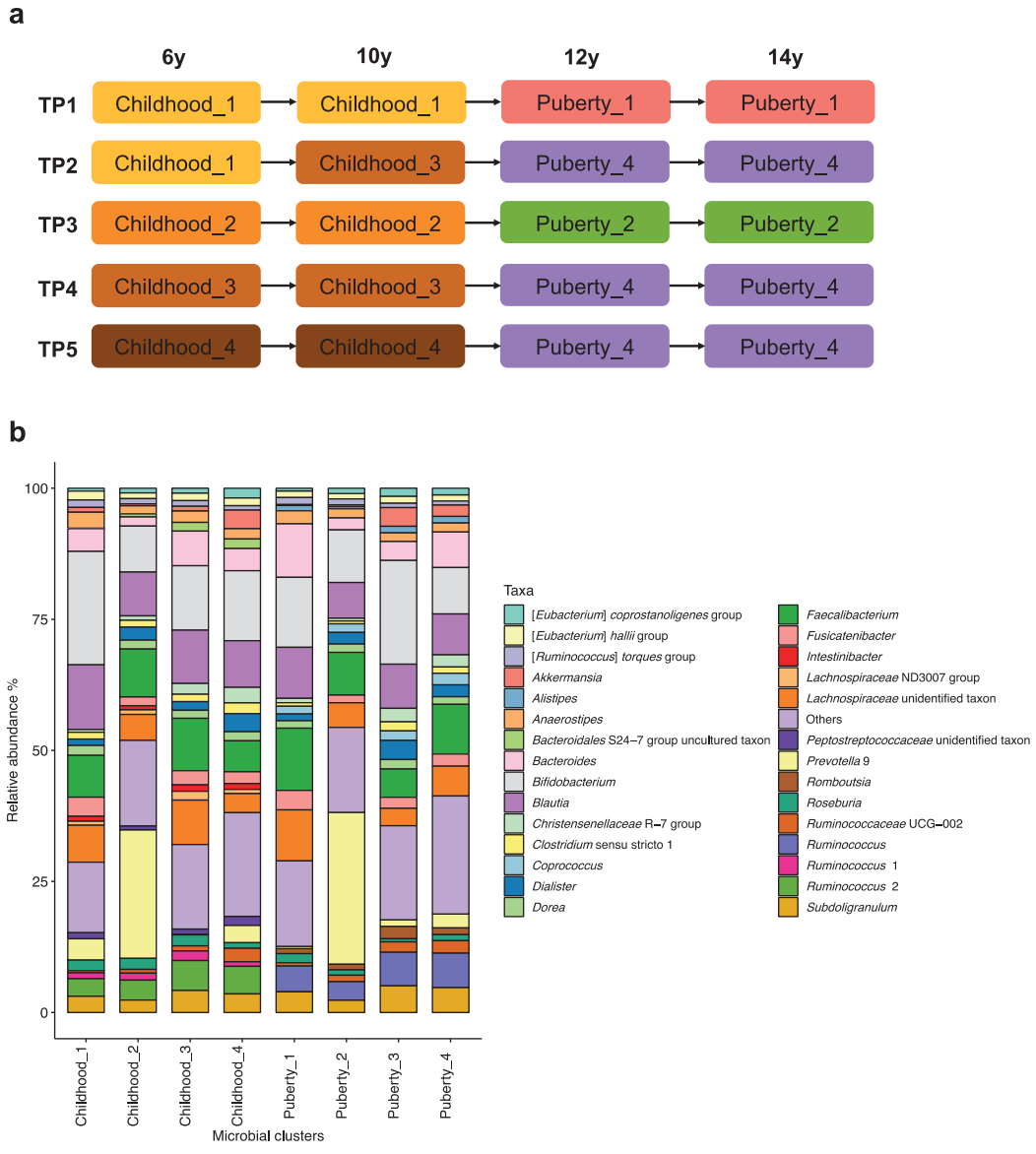

Figure 1. Features of microbial clusters in middle childhood and puberty. (a) transitional patterns of microbial clusters. TP1, TP2, TP4, and TP5 were potentially predominant transitional patterns with transitional rates higher than 10% between every two consecutive microbial clusters. TP3 was not a predominant transitional pattern but numerically enriched in *Prevotella* 9. (b) average relative abundances of microbial taxa at the genus level in microbial clusters in middle childhood (average over ages six and ten years) and puberty (average over ages 12 and 14 years). Others represent genus-level taxa with relative abundances lower than 1%.

Some compositional features were commonly shared between childhood and pubertal clusters within several of the transitional trajectories (Figure 1b). TP1 and TP5 were characterized by continuously low and high phylogenetic diversity, respectively. Furthermore, TP3 was enriched in *Prevotella* 9, a fiber-favoring but proinflammation-relevant taxon (Tett, Pasolli, Masetti, Ercolini, & Segata, 2021). In contrast, TP2 and TP4 exhibited transitional modes with more pronounced fluctuations. Interestingly, these two patterns converged to the same developing pattern at the age of ten to 14 years, although they started from different microbial clusters at the age of six years. However, it is not yet clear which specific factors drive such a notable shift and bring out subsequent consistency. To sum up, on the one hand, the stability of TP1, TP3, and TP5 suggests that some children may start establishing a stable gut microbiota ecosystem from middle childhood and maintain this stability when transitioning into puberty. On the other hand, the fluctuations in TP2 and TP4 imply that some children may maintain a more dynamic gut microbial ecosystem due to larger changes in environmental factors, either known or unknown (Falony et al., 2016; Gacesa et al., 2022; Rothschild et al., 2018; Zhernakova et al., 2016). Future studies may benefit from exploring how transitional trajectories of the gut microbiota are associated with environmental factors of the host, in particular whether factor strengths and durations would stimulate microbial changes in composition and functionality to a different extent.

Finally, one cluster in puberty, i.e., Puberty_3, failed to show the stability observed in all other pubertal clusters. Instead of staying at the same cluster, children within Puberty_3 exhibited divergent transitions from age 12 to 14. Additionally, Puberty_3 was numerically enriched in *Bifidobacterium*, a taxon thought to be abundant in early-aged children but decreasing till adulthood (Derrien, Alvarez, & de Vos, 2019), suggesting that Puberty_3 might reflect a more immature state. Replications are warranted to confirm these findings on predominant transitional patterns in healthy developing children.

Extended role of breastfeeding in shaping gut microbiota composition

The developing gut microbiota in middle childhood was associated with early-life breastfeeding (**Chapter 3**). This long-lasting relation with breastfeeding confirmed earlier prospective findings by Zhong et al. and Cioffi et al. in children at an average age of seven and 11 years, respectively (Cioffi, Tavalire, Neiderhiser, Bohannan, & Leve, 2020; Zhong et al., 2019). Together, this evidence implies an extended role of breastfeeding in forming gut microbiota composition. Note, however, that our longitudinal data showed that the variation in microbial composition explained by breastfeeding diminished from infancy to middle childhood, suggesting an enduring but declining relation between breastfeeding and the gut microbiota.

Notably, although statistical tests showed significant relations between breastfeeding and microbial composition in this thesis, breastfeeding only explained a moderate amount of interindividual variation in microbiota during infancy and middle childhood (adjusted R^2% = 0.556% and 0.329%, respectively). This is in line with previous findings (Stewart et al., 2018; Zijlmans, Korpela, Riksen-Walraven, de Vos, & de Weerth, 2015)

and with the results of several large-scale population studies, each comprising thousands of participants, that found no more than 20% of microbial variation to be collectively explained by environmental and genetic factors, with a large part of the variance in the gut microbiota remaining unexplained (Falony et al., 2016; Gacesa et al., 2022; Rothschild et al., 2018; Zhernakova et al., 2016). This "missing variance" may be attributed to additional factors that are currently unknown or undetectable.

The gut microbiota is related to behavior in low-risk community children

The interplay between the gut microbiota and brain functioning has been observed in many animal models and human case-control studies (Bundgaard-Nielsen et al., 2020; Cheung et al., 2019; Cryan et al., 2019; Hai yin Jiang et al., 2018; Sukmajaya, Lusida, Soetjipto, & Setiawati, 2021). However, it remains undetermined if the gut microbiota is related to the development of mental health in low-risk community children. In this thesis, we explored the relations between the gut microbiota and child behavior, based on both cross-sectional and longitudinal studies. Multiple potential microbiota-behavior links were unveiled herein. Among them, two genus-level microbial taxa, i.e., *Prevotella* 9 and *Phascolarctobacterium*, appear to stand out.

Prevotella 9 at the ages of six and ten years was positively related to mother- and child-reported externalizing behavior at the age of ten years (**Chapter 3**). Such positive links were also discerned between this taxon at the age of 12 years and mother-reported externalizing behavior at the same age (**Chapter 4**), and between *Prevotella* 9-enriched clusters in middle childhood and puberty and child-reported externalizing behavior at the age of 14 years (**Chapter 5**). A previous study reported increased *Prevotella* spp. at the age of one year being related to fewer internalizing difficulties at the age of two years (Loughman et al., 2020), whereas two other studies did not find such a relation in children (Laue et al., 2021; Van De Wouw et al., 2022). Divergent results of *Prevotella* spp. were also observed in children with mental disorders often characterized by externalizing behavior, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) (Bundgaard-Nielsen et al., 2020; De Angelis et al., 2013; Hai-yin Jiang et al., 2018; Kang et al., 2013; Li et al., 2022; Prehn-Kristensen et al., 2018; Wang et al., 2020). This discrepancy between findings might be due to large interindividual heterogeneity amongst studies and functionally-diverse species within *Prevotella* (Tett et al., 2021).

Phascolarctobacterium in middle childhood was positively associated with mother-reported externalizing behavior at age ten (**Chapter 3**), and a similarly positive link was found cross-sectionally between this taxon and child-reported externalizing behavior at age 14 (**Chapter 5**). To our knowledge, *Phascolarctobacterium* has not been positively related to externalizing problems previously (Laue et al., 2021; Loughman et al., 2020; Van De Wouw et al., 2022). However, two systematic reviews about ASD and major depressive disorder (MDD), found overgrowing *Phascolarctobacterium* in patients (Cheung et al., 2019; Iglesias-Vázquez, Van Ginkel Riba, Arija, & Canals, 2020), whereas one study consisting of three aircraft crew members, found more *Phascolarctobacterium* being linked to better mood

conditions (Li et al., 2016). Researchers have speculated that *Phascolarctobacterium* might affect outward-directed behavioral issues by producing the short-chain fatty acid propionate (Ikeyama et al., 2020). This speculation needs to be validated in future experiments.

Repeated consistent results at different time points reported in this thesis suggest *Prevotella* 9 and *Phascolarctobacterium* as potential taxa that participate in the microbiota-behavior interplay. Once our findings on these taxa can be confirmed in another similar cohort sufficient in sample size and bias control, we can make a step towards addressing underlying mechanisms and causal relations to behavioral conditions.

Moving from correlation to causation

As presented in this thesis, our observational studies uncovered multiple interesting correlations between the gut microbiota and child behavior. However, as the well-known phrase says, "correlation does not imply causation". It remains a great challenge to translate correlational findings into conclusive proofs of causality, especially along the human microbiota-gut-brain axis (MGBA). To add more insight on this axis, a workflow for exploring causal relations is introduced (Figure 2).

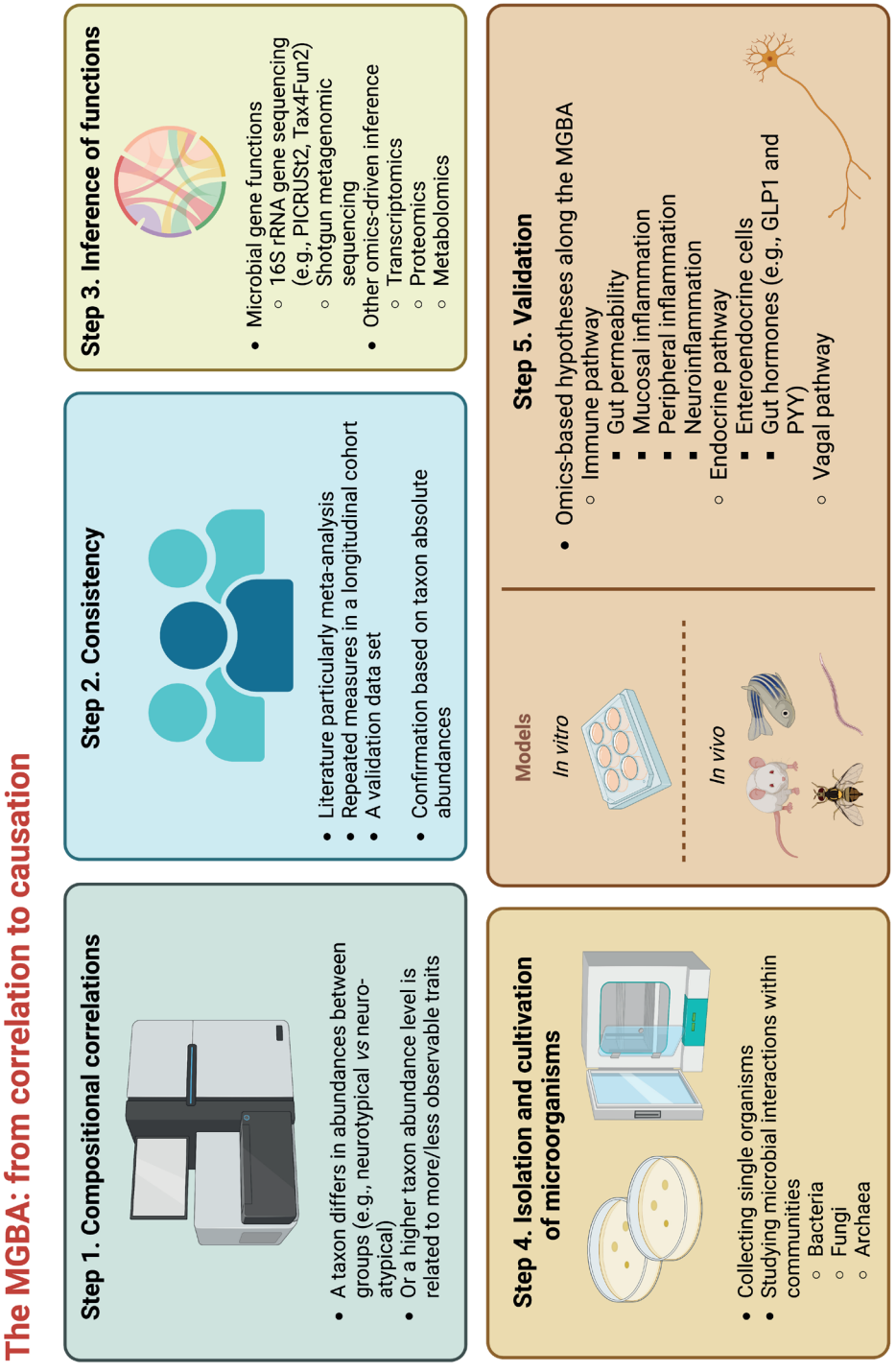

Figure 2. The workflow of shifting sequence-based correlative results into causality. MGBA, microbiota-gut-brain axis; GLP-1, glucagon-like peptide 1; PYY, peptide YY. (created with https://biorender.com/)

Step 1 shows two common types of microbial composition-based correlations, including differentially abundant taxa between groups and linear relations between taxa and observable traits. Although these correlations have been reported in an increasing number of studies, little convergence in correlation direction and strength has been reached till now.

Step 2 introduces several approaches to verify the consistency of the observed correlations. A useful strategy to synthesize diverse outcomes from the literature into a general opinion is meta-analysis. Meta-analyses have been conducted on behavioral profiles on which considerable evidence has been collected to date, such as on ASD (Bundgaard-Nielsen et al., 2020; Iglesias-Vázquez et al., 2020), MDD (Cheung et al., 2019), ADHD (Bundgaard-Nielsen et al., 2020; Sukmajaya et al., 2021), and temperament (Alving-Jessep, Botchway, Wood, Hilton, & Blissett, 2022). Although, to my knowledge, meta-analyses have not yet been applied to assessing microbiota-behavior links in community samples due to the limited number of studies, such analyses are highly recommended, once there are sufficient data. When meta-analyses are not feasible, the robustness of results within one study can be enhanced by performing repeated measures or including a validation data set.

Relative abundance-based correlations are widely used in describing microbial links to mental outcomes. However, relative abundance data have some inherent limitations, including increased correlational biases and false discovery rates. This may lead to an insufficient power to fully capture inter-individual variations in microbial composition (Barlow, Bogatyrev, & Ismagilov, 2020; Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020; Vandeputte et al., 2017). Therefore, instead of only using relative abundances, it is recommended to additionally include absolute abundances (or microbial load; measured by e.g., quantitative PCR or flow cytometry) when attempting to convert statistically significant findings into biological interpretations.

Once the consistency of the observed correlations is verified, we can give more attention to the inference of molecular mechanisms. To this end, an integrative use of omics-driven approaches is suggested in **Step 3**. Microbial gene functions can be the first accessible indicators for explaining the complex gut-brain interplay. For broadly used 16S rRNA gene sequence data, prediction tools, such as Picrust2 (**Chapter 3**), Tax4Fun2, and PanFP (Douglas et al., 2020, 2019; Jun, Robeson, Hauser, Schadt, & Gorin, 2015; Wemheuer et al., 2020), can leverage the data to the maximum. Although these prediction tools have been criticized for reference bias and limited resolution (Douglas et al., 2020), the increasing availability of reference data renders them more feasible alternatives to the still quite expensive shotgun metagenomic sequencing. Additionally, other omic techniques can be incorporated into the selection process of key pathways and possible biomarkers (Manzoni et al., 2018): (1) transcriptomics provides information on sample-specific gene expression features; (2) proteomics measures the entire set of proteins in target samples and therefore can be used to discover potential biomarkers; and (3) metabolomics studies metabolites in target samples and hence can help increase the understanding of relevant molecular pathways in specific conditions.

Before validating the inferred molecular mechanisms of candidate taxa, **Step 4** emphasizes the importance of isolation, cultivation, and characterization of specific

microorganisms. Availability of cultured representatives of target microorganisms is a prerequisite to meet the demand of experimental designs and even therapeutic strategies. A study in 2005 reported that approximately 80% of human gut bacteria have not been cultured yet (Eckburg, 2005). With high-throughput cultivation approaches being developed rapidly, it will be technically possible in the coming decades to produce personalized collections of gut microbial taxa with known genotypical and phenotypical characteristics (Clavel, Horz, Segata, & Vehreschild, 2022). In addition to collecting single microorganisms, it is also important to intensify research on studying interactions between various microorganisms: not only predominant bacteria, but also other microbes, such as fungi and archaea (Clavel et al., 2022). Fungi regulate gut immunity and are involved in gut-related diseases, such as inflammatory bowel disease, irritable bowel syndrome, and colorectal cancer (Richard & Sokol, 2019). After millions of years of coevolution, gut fungi and bacteria have developed various types of interactions, including mutualistic, commensal, and competitive relations (Richard & Sokol, 2019). Archaea in the human gut, mainly composed of methanogens, produce methane (i.e., a potential neuromodulator and immunoregulator) and affect host gut motility (Borrel, Brugère, Gribaldo, Schmitz, & Moissl-Eichinger, 2020). Also archaea interact with bacteria in the gut by utilizing bacteria-derived products and consuming hydrogen which improves energy yield and shifts metabolic outcomes (Borrel et al., 2020). These complex interactions between host, bacteria, fungi, and archaea constitute important challenges, but also underline the value of efforts aimed at obtaining a more detailed picture of these dynamic interactions. Only then will we be able to determine more precisely how different microorganisms influence host phenotypes.

Step 5 presents currently available *in vitro* and *in vivo* models used in validating pathways (e.g., immunity, endocrine system, and vagus nerve as three main pathways) along the MGBA (Cryan et al., 2019; Morais, Schreiber, & Mazmanian, 2021). Depending on study designs, different *in vitro* and *in vivo* models can be selected, such as organoids and animals (e.g., rodents, zebrafish, fruit fly, and nematodes), respectively (Cryan et al., 2019; Horvath et al., 2022; Nagpal & Cryan, 2021). For instance, organoids are self-organized three-dimensional tissue constructs that show *in vivo*-like structure and regional specification (Moysidou & Owens, 2021). To model microbial interactions with gut organoids, microbes or microbe-derived metabolites are injected into the inner part of organoids, and morphological and physiological traits of organoids are determined (Moysidou & Owens, 2021). Additionally, *in vivo* rodent models mimic potential causes and phenotypic outcomes of certain mental disorders (e.g., ASD, and depression and anxiety disorders), adding invaluable credits to causality exploration (Nagpal & Cryan, 2021; Nestler & Hyman, 2010). For example, probiotic *Lactobacillus reuteri* was applied to specific-gene mutant rodent models with behavioral deficits, and this taxon rescued social deficits and improved oxytocin levels (Nagpal & Cryan, 2021). According to the FAO/WHO definition, a probiotic strain must be (1) sufficiently characterized, (2) safe for the intended purpose, (3) supported by at least one human clinical study, and (4) alive at an adequate amount during shelf life (Binda et al., 2020). Although not all candidate taxa may seem qualified probiotics, metabolites derived from them may be interesting biomarkers or even drugs for various mental disorders.

Despite the feasibility given by model organisms in exploring causality, it is important to reiterate that observable behavioral traits are different between model organisms and human beings, which to some extent impedes the translation from bench to bedside (Cryan et al., 2019). For this reason, well-established validity standards must be applied to animal studies beforehand (Morais et al., 2021). Moreover, it has to be noted that the gut microbiota is a highly complex and interactive consortium, and studies of this community should not be restricted to specific microorganisms. How microbes interact with each other and jointly influence host phenotypes at a molecular level is an essential part of the puzzle that should receive more attention over the coming years.

Challenges in selecting confounders

For observational studies aiming to infer potential causal relations, it remains a major concern to reduce confounding effects (VanderWeele, 2019). Confounders are variables that influence both predictor and outcome variables. It is not a simple endeavor to identify confounders in studies focused on associations between complex systems with numerous variables (e.g., the gut microbiota and host behavior), as knowledge about the relations between these variables is often insufficient and unavailable.

In the following paragraphs, I present several considerations for choosing confounders, with the goal of inspiring the field:

(1) It is common in this field to choose potential confounders by referring to what has been previously reported in the literature. However, for the studies presented in this thesis, the current scarcity of research about associations of the gut microbiota with problem behavior meant that very few references were available for confounder selection. Also, as the gut microbiota and behavior are sensitive to many variables (known *vs* unknown; detectable *vs* undetectable), it is nearly impossible to include all of them. To visualize such complex relations, a directed acyclic graph (DAG) can be helpful, as it provides insight into variables that have to be accounted for (Textor, Hardt, & Knüppel, 2011). Criteria to identify such variables with the use of DAGs have been elaborated by Cinelli et al. (Cinelli, Forney, & Pearl, 2020). In microbiota research, Eckermann et al. used a DAG to graphically describe potential confounders of the relation between the gut microbiota and executive functions. This in turn provided a strong rationale for choosing confounders (Eckermann, Ou, Lahti, & de Weerth, 2022).

(2) When assessing a confounding effect, statistical significance is often determined based on a simple *p* value. The *p* value is used to decide whether to accept or reject the null hypothesis. Although being widely adopted so far, more and more researchers have called for an end of simply using such a conventional and dichotomous way when declaring if an outcome rebuts or supports a hypothesis (Amrhein, Greenland, & McShane, 2019). Instead of being overdependent on a *p* value, more attention should be given to a confidence interval (or a credible interval), which provides the range of plausible values of a relation (Schober & Schwarte, 2018).

(3) Collinearity can happen when two or more variables are strongly inter-related. Although researchers are aware of this phenomenon, the degree of collinearity has not been

frequently reported in previous microbiota studies. Including confounders with high collinearity levels can distort the interpretation of outcomes, and for this reason pre-checking collinearity is needed.

(4) Presenting both crude relations without confounders and adjusted relations with confounders is a common practice in epidemiological research (Kong, Zhang, Cao, Mao, & Lu, 2020; Verkouter et al., 2019; Vissing, Chawes, Rasmussen, & Bisgaard, 2018). This provides information about how confounders influence associations and increases the interpretability of outcomes. For this reason, it is advisable to show both relations when studying microbiota-behavior links.

In sum, there is no gold standard method for confounder selection and no consensus on which confounders have to be included in studies linking gut microbiota to behavior in children. As a consequence, different studies often comprise a varied set of confounders, making comparisons and meta-analyses often hard to implement. Following the suggestions presented above can help improve the solidity and comparability of the results of this research field.

Exploring the MGBA through integrative analytical approaches

The gut microbiota is a highly complex system. Compositional analysis by amplicon sequencing generates a vast amount of data, which are usually high-dimensional, phylogenetically-structured, zero-inflated, and over-dispersed (Chen & Chen, 2018). These microbial features pose great difficulties when examining microbial communities. Using a suitable method that can better handle such features can improve the interpretation of outcomes. In the following, I discuss the pros and cons of several complementary and sophisticated biostatistical approaches, used to explain microbial relations to behavioral measures in this thesis: (1) constrained methods such as redundancy analysis (RDA), (2) random forest algorithm (RF), (3) the framework of Dirichlet multinomial mixtures (DMM), (4) generalized linear models (GLMs), and (5) Bayesian linear models.

RDA directly shows how much variation in microbial composition is explained by behavioral measures. By drawing a triplot including samples, microbial taxa, and behavioral measures, we can deduce which taxa fit an RDA model the best and how taxa are potentially related to mental outcomes. This then provides information for follow-up validations of specific taxa. However, as RDA assumes linear relations between microbial data and behavioral measures, it is not suitable to explain complex non-linear relations. As a more appropriate alternative, another constrained ordination analysis, i.e., canonical correlation analysis (CCA), can be used for analyzing unimodal relations.

Compared to RDA (or CCA) models, RF models can identify both linear and non-linear relations between microbial data and behavioral outcomes. However, RF models work best with a large number of samples, and hence their results must be interpreted with caution when sample size is limited. When working with adequate samples, RF models provide useful information regarding the importance of specific microbial taxa, and permit selection of relevant taxa for downstream validations.

DMM can compress complex high-dimensional microbial data into a simplified low-dimensional matrix and is therefore considered to be a useful tool in identifying microbial patterns with different compositional features. This can largely facilitate the comparisons of mental outcomes between compositional patterns. However, it is important to note that reduction of dimensionality increases the risk of unexpectedly losing relevant information in the data.

In addition to the three multivariate analytical approaches aforementioned, GLMs and Bayesian linear models can be used to explore univariate relations between single microbial taxa and behavioral measures, which have been used in our research and previous studies (Hermes, Eckermann, de Vos, & de Weerth, 2020; Valles-Colomer et al., 2019). In general, running a GLM is quicker and computationally less demanding compared to running a Bayesian linear model. However, Bayesian models outperform GLMs in several aspects (Dunson, 2001): (1) using a posterior distribution as an alternative to a *p* value; (2) able to incorporate previous information from literature by including a prior probability distribution; and (3) extreme flexibility in straightforwardly fitting models to a complex data set with missing observations and multidimensional outcomes. Using these models can help shed light on specific taxa that have the potential of being key biomarkers.

Note that single models may never adequately represent all aspects of the highly complex MGBA. For this reason, an integrative use of analytical approaches in exploring the MGBA in observational studies appears highly advisable. Up till now, an increasing number of techniques have been developed to achieve specific goals in the field of the gut microbiota. One major goal is the identification of differentially abundant microbial taxa between different groups of participants. For this aim, methods such as LEfSe (Linear discriminant analysis Effect Size), MaAsLin2 (Microbiome Multivariable Associations with Linear Models), ANCOM (Analysis of Composition of Microbiomes), and ALDEx2 (ANOVA-like Differential Expression analysis), have been designed. However, determinations of differentially abundant taxa can vary drastically between methods due to varying concepts, algorithms, and requirements, and hence it is necessary to consider such discrepancy when comparing findings between studies (Nearing et al., 2022). Moreover, due to a recent growing body of longitudinal microbiota cohorts, longitudinal methods have been developed to capture both intra-individual dynamics and inter-individual differences between groups of interest (Kodikara, Ellul, & Lê Cao, 2022). For example, a time-course gene set analysis has been developed and is able to detect a change of a group of genes over time (Hejblum, Skinner, & Thiébaut, 2015). In 2021, Roswall et al. implemented this time-course analysis to a longitudinal child cohort and distinguished four microbial developmental trajectories from birth to the age of five years (Roswall et al., 2021). However, to date, longitudinal methods have not been frequently applied to real microbiota data, and their performance awaits to be validated. Summarizing, to obtain the most thorough description and information-rich view of the MGBA in observational studies, it is highly recommended to implement multiple complementary and sophisticated statistical approaches.

Concluding remarks and future perspectives

As reflected in this thesis, the development of the gut microbiota is a long-term process. Numerous factors, both known (explaining no more than 20% of microbial variation) and unknown, shape gut microbial communities to adapt to the ever-changing environment during sensitive time windows (Falony et al., 2016; Gacesa et al., 2022; Rothschild et al., 2018; Zhernakova et al., 2016). The results reported here showed that as a consequence of these factors, the gut microbiota, represented by microbial clusters with varying compositional features, developed in different trajectory patterns from birth till the age of 14. The frequency of inter-cluster transitions decreased distinctly in puberty compared to the first ten years of life, likely suggesting that a stable gut microbiota is consolidating at this age. Although our results regarding gut microbiota development are based on relatively large-sized community samples followed over an extended period, it is important to confirm our findings in other even larger populations in the future. A design including repeated measures of the gut microbiota is also advisable, as day-to-day variations of microbial relative and absolute abundances appear to be substantially larger within individuals than between individuals (Vandeputte et al., 2021). Furthermore, a more frequent use of whole-genome shotgun metagenomic sequencing is recommended, due to its enhanced detection of microbial taxa at the species level and improved prediction of microbial gene functions (Ranjan, Rani, Metwally, McGee, & Perkins, 2016). Additionally, instead of overreliance on relative abundances (characterized by some inherent limitations, such as more correlational biases, high false discovery rates, and the insufficiency to describe inter-individual differences), absolute abundances should be considered more often in future microbiota research (Barlow et al., 2020; Jian et al., 2020). Moreover, a time-course analysis may be an ideal approach to distinguish developmental trajectories of the gut microbiota in a longitudinally-designed study (Hejblum et al., 2015; Roswall et al., 2021).

In the current thesis, breastfeeding was found to show a long-lasting relation to the gut microbiota. In addition to feeding types, factors such as age, delivery mode, introduction of solid food, also explained microbial variance, albeit only to a limited extent. It is noteworthy that more than 80% of microbial variance cannot yet be explained by known environmental and genetic factors, as concluded in large-scale population research (Falony et al., 2016; Gacesa et al., 2022; Rothschild et al., 2018; Zhernakova et al., 2016). This, together with a lack of knowledge on the nature of interactions between the gut microbiota, and between the microbiota and the environment, indicate a necessity to deeply explore such microbial interplay.

To conclude, main contributions of this thesis were the associations found between the gut microbiota and behavioral development in community children from birth to 14 years of age. Nevertheless, it is important to stress the fact that correlation does not imply causation. At this highly exploratory stage of the field, few studies exhibit consistency in directions of such relations. Therefore, the first priority is to carry out bias-controlled replication studies to reach consensus on the type and direction of associations. Once consistency is determined, more attention can be given to causal relations. To infer mechanistic causations, it is advisable to adopt integrative omics-driven approaches, such as

metagenomics for predicting gene functions and metabolomics for profiling functional metabolites. These approaches require strong bioinformatic and biostatistic support. The selected gene functions and potential functional metabolites can then be carefully validated in suitable *in vitro* and *in vivo* models (Cryan et al., 2019; Horvath et al., 2022; Nagpal & Cryan, 2021). As I summarized earlier, although relative abundances are useful in describing microbial compositional features, more biologically insightful interpretations regarding the MGBA can be acquired through absolute abundances (or microbial load). Finally, yet importantly, the gut microbiota is a highly complex and interactive ecosystem, and for this reason future research should not only focus on a specific microbe but also look at how microbial interactions jointly influence host mental development and health.

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Summary

The human gut is inhabited by a huge number of microorganisms that are intimately involved in many fundamental aspects of host fitness. In the past two decades, increasing attention has been paid to the role of microorganisms in the microbiota-gut-brain axis. Through this axis and during sensitive periods such as infancy and puberty, the gut microbiota may hold more conspicuous relations to the gradually maturing host brain than at any other time point. The research described in this thesis focused on (1) the development of the gut microbiota as well as factors related to this development from birth to puberty, and on (2) how the gut microbiota was associated with child mental development and health in two longitudinal Dutch community cohorts (BINGO and BIBO).

Chapter 1 first briefly introduced the gut microbiota, touching upon its definition, relevant determining factors, development in early ages, and importance in health and disease, in particular its potential links to cognition and problem behavior in infancy and childhood. Furthermore, this chapter introduced experimental approaches used in deciphering gut microbiota composition and statistical methods used in disentangling microbial relations to host observable traits.

Chapter 2 explored relations between the gut microbiota and problem behavior and executive functions in the first three years of life in the BINGO cohort. By performing age-specific and time trajectory analyses, multiple associations were discerned. Increased relative abundance of the genus *Streptococcus*, specifically at age two weeks and generally over the first three years, was related to worse performances in executive functions at age three years. Furthermore, relative abundance of [*Ruminococcus*] *torques*, a group that has been linked to inflammation, was inversely related to internalizing difficulties at age three years and over the period from age one to three years. In addition, three robust age-specific relations were observed: higher relative abundances of *Bifidobacterium* at age three years were linked to more problem behavior at the same age; higher *Blautia* relative abundances were associated with fewer internalizing difficulties in aged-three children; and increased relative abundances of an unidentified taxon within the *Enterobacteriaceae* family at age two weeks were related to more externalizing behavior at age three years. Moreover, evidence was found that higher alpha diversity at age two weeks was related to fewer internalizing problems and better executive functions at age three.

Chapter 3 described transition patterns of developing gut microbiota in the first ten years of life in children of the BIBO cohort. Three distinguishable microbial community patterns were identified in infancy, and another four microbiota patterns were pinpointed in childhood via clustering analyses. One infant microbial cluster increased in prevalence with age, and two childhood microbial clusters became more widespread from age six to ten years. Regarding compositional features, two infant microbial clusters were predominated by *Bifidobacterium*, and one childhood cluster was strongly enriched in *Prevotella* 9, resembling a human enterotype seemingly independent of age. Notably, breastfeeding contributed to variation in microbiota composition up to age ten, implying a potential enduring influence on gut microbial ecology. No associations were observed between microbial clusters and problem behavior in this study. However, we found that increased

relative abundance of *Prevotella* 9 in childhood was related to more mother-reported child externalizing behavior at age ten, which was further verified by child reports.

In **Chapter 4**, we designed a cross-sectional study for twelve-year-old BIBO children. This study explored gut microbiota compositional differences between boys and girls at the onset of puberty, and investigated gut microbiota composition as well as microbiota-derived metabolites in relation to child behavior, also taking gender into account. Importantly, both relative and absolute abundance data of the gut microbiota were included. We observed subtle gender-specific differences in gut microbiota beta diversity but not in alpha diversity or individual taxon abundances. Regarding microbial relations to child behavior, *Ruminococcaceae* UCG 004 and *Parasutterella* displayed positive relations to mother-reported internalizing behavior, being consistent between relative and absolute abundance measures. Also, increased *Odoribacter* was related to less mother-reported externalizing behavior, and increased *Parasutterella* was associated with less child-reported prosocial behavior. Moreover, we detected a positive trend between *Prevotella* 9 and mother-reported externalizing difficulties, confirming our earlier findings on the same community group in middle childhood. Finally, *Parasutterella*, *Coprococcus* 3, and *Ruminococcaceae* UCG 003 showed gender-dependent relations to internalizing, externalizing, and prosocial behavior, respectively. Concentrations of fecal short-chain fatty acids and branched-chain fatty acids did not exhibit relations to any behavioral measures. Except for valerate and isovalerate, no gender-related differences were observed in associations between microbiota-derived metabolites and behavioral scales.

In **Chapter 5**, to extend our knowledge on long-term microbial relations to child behavior, we linked the gut microbiota in the first 14 years of life to problem behavior and social anxiety at age 14. First, we delineated gut microbiota development over this period, by incorporating microbial clusters that were reported in Chapter 3 with newly-identified ones at the age of 12 and 14 years. Four distinct microbial clusters were observed in puberty, three of which resembled age-independent enterotypes in compositional features. Most children within these three clusters stayed in the same clusters from age 12 to 14, implying stability in microbial development and transition during this period. Two *Prevotella* 9-predominated clusters, including one in middle childhood and the other one in puberty, showed more externalizing behavior at age 14. Additionally, children from one *Faecalibacterium*-depleted cluster in puberty exhibited more difficulties regarding social anxiety at age 14. This was confirmed by a negative relation between *Faecalibacterium* and social anxiety in aged-fourteen children.

Chapter 6 discussed the main findings of this thesis and the challenges encountered. Our studies depicted continued development of the gut microbiota from birth to puberty and shed light on latent relations between the gut microbiota and child mental development and health during sensitive time windows. To translate our correlation-based results into causality, these findings must be first carefully verified in other bias-controlled research. Once consistent outcomes are achieved, further inference on underlying mechanisms can be achieved by incorporating omics-driven approaches. Such analyses might point towards

specific pathways along the microbiota-gut-brain axis that can be targeted for ensuing validations in suitable model organisms.

Appendices

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About the author

Yangwenshan Ou was born on the 15th of February 1994 in Beijing, China. She was raised in Beijing and obtained her bachelor's degree of food science and engineering at China Agricultural University (CAU). Then, she continued studying food science as a master's student in 2016 at the Key Laboratory of Functional Dairy at CAU. During her master's study, Yangwenshan was fascinated by research about the role of the gut microbiota in human health and disease. Therefore, she performed her master's thesis by assessing the alleviating effects of probiotic beverage Yakult on Chinese constipated patients, under the supervision of Dr. Liang Zhao and Dr. Fazheng Ren. This human study uncovered several fecal metabolites relating to constipation improvement, of which the most differentially abundant one was verified by her in an animal study later. After obtaining her master's degree in 2018 at CAU, Yangwenshan received financial support from China Scholarship Council to work as a PhD at the Laboratory of Microbiology, Wageningen University & Research (WUR), the Netherlands. Under the supervision of Dr. Clara Belzer (WUR), Dr. Hauke Smidt (WUR), and Dr. Carolina de Weerth (Radboud university medical center), she depicted gut microbiota development in children from birth to age 14 and explored microbial relations to child mental health, as described in this thesis.

List of publications

Chen, S., **Ou, Y.**, Zhao, L., Li, Y., Qiao, Z., Hao, Y., & Ren, F. (2019). Differential effects of *Lactobacillus casei* strain Shirota on patients with constipation regarding stool consistency in China. Journal of Neurogastroenterology and Motility, 25(1), 148–158. https://doi.org/10.5056/jnm17085

Eckermann, H. A., **Ou, Y.**, Lahti, L., & de Weerth, C. (2022). Can gut microbiota throughout the first ten years of life predict executive functioning in childhood? Developmental Psychobiology, 64(3), 1–14. https://doi.org/10.1002/dev.22226

Ou, Y., Belzer, C., Smidt, H., & de Weerth, C. (2022). Development of the gut microbiota in healthy children in the first ten years of life: associations with internalizing and externalizing behavior. Gut Microbes, 14(1). https://doi.org/10.1080/19490976.2022.2038853

Ou, Y., Chen, S., Ren, F., Zhang, M., Ge, S., Guo, H., … Zhao, L. (2019). *Lactobacillus casei* strain Shirota alleviates constipation in adults by increasing the pipecolinic acid level in the gut. Frontiers in Microbiology, 10(FEB), 1–12. https://doi.org/10.3389/fmicb.2019.00324

Overview of completed training activities

Discipline specific activities	Organizing institute(s)	Year
Gut Day	WUR	2018
Early Programming: How Early Life Shapes Human Development	Radboud University	2019
Gut Day	WUR & University Medical Center Groningen	2019
Intestinal Microbiome of Humans and Animals	VLAG	2019
ICIS	International Congress of Infant Studies	2020
Mini symposium: In vitro studies of the human intestinal microbiota	WUR - Laboratory of Microbiology	2020
SRCD 2021 Biennial Meeting	Society for Research in Child Development	2021
The Microbiome: From Mother to Child	Keystone Symposia	2021
The International Conference on Microbiota-Gut-Brain Axis	Mind Mood & Microbes	2021
Gut-Brain Axis	Keystone Symposia	2022
Annual KNVM Meeting	KNVM	2022
International human microbiome consortium (IHMC) 9th congress	IHMC committee	2022
General courses	**Organizing institute(s)**	**Year**
Presenting with Impact	Wageningen in' to Languages	2019
Scientific Writing	Wageningen in' to Languages	2019
Introduction to 'R'	VLAG	2019
Research Data Management	Wageningen University& Research Library	2019
Project & Time Management	Wageningen University& Research	2019
PhD week	VLAG	2019
Other activities	**Organizing institute(s)**	**Year**
Preparing Project Proposal	VLAG	2018-2021
MolEco Group Meeting	WUR - Laboratory of Microbiology	2018-2022
MIB PhD Meeting	WUR - Laboratory of Microbiology	2018-2022
Journal Club	WUR - Laboratory of Microbiology	2018-2020
DPB Group Meeting	The Developmental Psychobiology Lab	2018-2022
Assisting in teaching and supervision activities		**Year**
Course MIB 30303 (Research Methods Microbiology)		2019-2022
Supervising student(s)		2021-2022

Colophon
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