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How microbiota composition and diversity are related to striatal volumes in early life.

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Master's thesis

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A primary goal of gut-brain research is to understand how human gut microbiota affect mental health such as cognition or neuropsychiatric disorders. Studies employing rodent models reported that gut microbiota had a strong influence on neurodevelopment, especially during the critical period of 'developmental programming', which seems to be a key window for external influences. However, knowledge of the origins of the relationship between gut-microbiota and brain functions is still limited due to the scarcity of the human gut-brain studies, particularly those focusing on infant populations. Deep brain structures, in particular subcortical structures such as the striatum, are major components to the gut-brain axis and also have been shown to have a central role in neurodevelopment. Finally, previous literature demonstrates that the first years of life are critical to neurodevelopment and gastrointestinal colonization.

The aim of this thesis is to explore the relationship between the fecal microbiota composition and diversity of 2.5 months infants and the striatum volumes measured at one month. This exploratory cross-sectional study was conducted using data collected on 56 infants drawn from the FinnBrain Birth Cohort study, which focuses on the effects of environmental and genetic stimuli on child development and health.

The results showed no statistically significant associations between alpha and beta-diversity, and the striatum volumes. The correlation between alpha-diversity and striatum volumes tended to present visible sex-differences, despite their statistical insignificance. This novel study encourages future longitudinal studies to provide insight into the relationship between characteristics of the gut microbiota and the development of the brain especially during the critical phases of neurodevelopment throughout life.

Key words: infant; gut; microbiota; gut-brain axis; striatum.

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1 Introduction

We live symbiotically with a community of microorganisms (bacteria, archaea, viruses, phages, yeasts and fungi) inhabiting various parts of our body. The gut microbiota, in our colon, is one of the best examples of these symbiotic relationships. (Cani, 2018; Cryan et al., 2019). It is known to have a central role in our general health, as it interacts with virtually all of the cells present in our body, including those of the nervous system (Cani, 2018). Besides, gut microbiota contributes to several essential metabolic and trophic functions and to processes involved in organism protection from infections (Guarner & Malagelada, 2003). The existence of the gut-brain axis was first evidenced in the late nineteenth century by the pioneering work of Carl Lange and William James, who proposed that signals from the viscera to the brain modulated directly some emotional responses (Eisenstein, 2016). Over the past decades, our understanding of the gut microbiota's influence on the human brain (e.g., metal health or neurodevelopment) broadened and the microbiota-gut-brain research received increasing interest. This young and fast-growing area of research questions previous paradigms about human capacities by looking at the bidirectional communication between the gut and the brain (Hooks et al., 2018; Mayer et al., 2014).

At present, the main focus of gut-brain research is the investigation of the relationship between the microbiota and various aspects of mental health (Christian, 2019) such as stress processes, cognition and mood, or neuropsychiatric disorders (Hooks et al., 2018; Mayer et al., 2014). Moreover, the gut microbiota has been shown to have a role in normal neurological, cognitive and social development (Desbonnet et al., 2014; Carlson et al., 2018; Fröhlich et al., 2016). However, major advances in understanding the microbiota-gut-brain were made during research on rodents' models (Sudo et al., 2004; Neufeld et al., 2011), thus leading to a scarcity of human-based resources (Hooks et al., 2018; Cryan et al., 2019; Carlson et al., 2018).

Research to date showed that gut microbiota contributes to the phenomenon known as 'developmental programming' of an organism, especially for the brain. Developmental programming is defined as the modulation of a process impacted by environmental factors during a sensitive developmental window that has long-term structural and functional effects on an organ (Heijtz et al., 2011). Both gastrointestinal microbiota colonization and neurodevelopment occurs primarily during the first years of life providing opportunities for interactions such as developmental programming. Indeed, the gut microbiota resembles its

adult status in humans by the age of three, while foundational neurodevelopment processes occur over the first two years of life (Carlson et al., 2018; Gilmore et al., 2012; Knickmeyer et al., 2008; Provost, Hanganu, and Monchi, 2015). The close relationship between gut microbiota and brain development has been notably demonstrated in germ-free rodent studies, which showed that microbiota can have a long-lasting impact on neurodevelopment (Heijtz et al., 2011; Sudo et al., 2004). Furthermore, in their study, Neufeld et al. (2011) demonstrated that the effects of early disruption of the rodent microbiota establishment might persist even after restoring the microbiota using recolonization treatments at the adult stage.

The ability of gut microbiota to influence neurodevelopment has been highly studied in rodents but only sparsely in humans (Hooks et al., 2018; Cryan et al., 2019; Sudo et al., 2004). The findings of these clinical studies indicate that gut microbiota diversity correlates with temperament, cognitive development and functional connectivity during infancy (Aatsinki et al., 2019; Carlson et al., 2018; Kelsey et al., 2019; Yang et al., 2016; Gao et al., 2019). A highly diverse microbiota is commonly associated with good health; however, this idea has been challenged (Shade, 2017; Hooks et al., 2018). Indeed, a study by Carlson et al. (2018) revealed that higher levels of bacteria diversity – measured as intra-individual diversity (alpha diversity) – was not invariably associated with cognitive development benefits in infants. It should be noted that infants' diet is relatively homogenous (breastmilk or formula) and leads to a relatively low microbiota diversity. Indeed, gut microbiota diversity increases upon weaning and diversification of diet (e.g., solid foods) and motor development (e.g., crawling or walking) which extends the infant's environmental exposures (Borre et al., 2014). In all, this suggests that gut microbiota diversity during infancy may have greater, but ambiguous, associations with later development compared with adults.

The deep brain gray matter nuclei, such as the basal ganglia, play a central role in the complex networks regulating our behaviours (Provost, Hangaru et Monchi, 2015). The basal ganglia, and more specifically their main component the striatum, are essential in the functioning mechanisms of the brain's networks for both motor and non-motor functions (Mink & Thach, 1993; Provost, Hangaru et Monchi, 2015). The striatum more specifically has been linked to the motor and reward systems (Provost, Hangaru et Monchi, 2015; Mink & Thach, 1993). Many neuropsychiatric or neurologic diseases involve the disturbance of the striatum's normal functions (e.g., autism, ADHD, Huntington, schizophrenia, Parkinson's disease) (Pitenger & Duman, 2008; Liu et al., 2017; Hanganu, 2015; McCutcheon et al., 2019). Therefore, it is essential to understand the physiology behind the striatum normal and

pathological functioning, as it may be vulnerable at various times in life to external factors. Hence, the gut microbiota is an external factor that might affect the networking of the striatum, particularly in the sensitive window of early life.

A large body of research targeting the gut-brain axis and its effect on neurodevelopment involves preclinical animal model studies and clinical studies on the adult population. Many more clinical studies are essential to show the implication of gut microbiota in the striatum's structural and functional development. It has been suggested that early life is a sensitive period with a higher vulnerability to external factors, similar to the microbiota on the behaviour (Sudo et al., 2004). For this reason, studies on children, and even more so on infants, are essential to examine the origins of the role that early gut microbiota diversity plays in the structural development of the brain and in particular in the striatum.

1.1 Microbiota-Gut-Brain Axis

Studies of the gut microbiota have shown that the bacterial cells' genetic material outnumbers human genetic material by a hundred-fold (Gilbert et al., 2018). Notably, the gut microbiota affects major physiological processes, including immune system functions or the biosynthesis of multiple molecules (e.g., vitamins, neurotransmitters) (Huttenhower et al., 2012; Koenig et al., 2011) and the metabolism of toxins and drugs (Rekdal et al., 2019). Hence, it is crucial to understand the gut microbiota development and how it communicates with the central nervous system.

1.1.1 Developmental windows for the gut and brain developments

Early life is a turbulent time for development, and prenatal and postnatal periods are crucial developmental windows characterized by rapid and considerable evolution in the neuronal and microbial organization. Brain development involves an intricate equilibrium between genetic and environmental factors, that if disrupted may have long-term impacts on the brain and individual behaviour later in life (Borre et al., 2014). The nervous system undergoes a coordinated series of temporally regulated neurodevelopmental events pivotal for functional, cognitive, motor and emotional development. Neurodevelopment begins *in utero* and evolves during postnatal development and throughout the lifespan (Ben-Ari, 2013; Borre et al., 2014; Workman, 2013). Presumably, early life disturbances during gut microbiota development could impact neurodevelopment and health outcomes later in life (Borre et al., 2014).

Prenatal development of the microbiota and the brain

The initial establishment of the gut microbiota is a crucial step in neonatal development, although its early timing contradicts the current dogma that the fetal and intrauterine environments are sterile (Aagaard et al., 2014; Kuperman et al., 2020; Olomu et al., 2020; Rackaityte et al., 2020). The suggested *intrautero* microbial colonization may result from a microbial translocation from the mother to the fetus via the placenta and bloodstream. Several preclinical and clinical studies have demonstrated the presence of a bacterial signature similar to that of the mother's oral cavity, in the umbilical cord blood, placenta, amniotic fluid, and meconium (Funkhouser & Bordenstein, 2013; Jiminez et al., 2005; Aagaard et al., 2014; Borre et al., 2014). However, the idea of *intrautero* colonization is debated, as detection of bacterial DNA does not establish a causal link proving fetal gut microbial colonization (Koren et al., 2012; Perrez-Muños et al., 2017). Rather, it should be thought of as fetal microbial exposures which may participate, for example, in immunological programming of the fetus (Buffington et al., 2016). Of significance, this intrauterine fetal microbial exposure window, and potential gut colonization, is overlapping with the intricate prenatal window for brain development.

Brain development begins as early as 3-4 weeks of gestation with the phenomenon of neurulation. This first significant neurodevelopmental process leads to the formation of the neural tube critical to normal development (Rice & Baronne, 2000; Stilles et al., 2010). Next, neurogenesis, gliogenesis and synaptogenesis conduct to the formation of neurons, glial cells, and synapses, respectively. These processes are predominant during gestation but can last up to late infancy (2.5 years of age), especially synaptogenesis (Workman et al., 2013, Stilles et al., 2010). The concomitant developmental phases of neurons, glia and synapses formation insures the appropriate establishment of neuronal-glial interactions (Barker & Ullian, 2010). Furthermore, the developing brain is highly vulnerable to both internal and external environmental influences. Maternal health plays a crucial role in microbiota and brain development and characterization, as it vectors internal and external cues affecting fetal development. Indeed, prenatal maternal diet, infection and stress have been associated with certain neurodevelopmental disorders such as autism and attention deficit hyperactivity disorder (ADHD) (Donnet-Hughes et al., 2010; Bale et al., 2010). Hence, during this sensitive window for brain development, maternal microbiota translocation might have an incidence on neurodevelopmental disorder risk later in life.

Postnatal development of the microbiota and the brain

The gut microbiota is inoculated at birth, but undergoes significant changes in its composition at the introduction of solid foods and weaning (Stewart et al., 2018; Bäckhed et al., 2015). During birth, infants are exposed to microbes originating from their mother and, toa lesser extent, from external environment. The inoculation and subsequent development of the gut microbiota in early life are crucial for health outcomes, particularly neurodevelopment (Stewart et al., 2018; Bäckhed et al., 2015; Borre et al., 2014). The diversity of the microbiota is at first low and unstable. However, many factors may influence the gut microbiota's composition and its functions during this sensitive period. Birth mode, gestational age, and genetic and environmental factors (e.g., feeding mode) are key factors influencing the gut microbiota's initial composition (Borre et al., 2014). The introduction of solid food and weaning leads to a significant change in the gut microbiota composition as diet is a crucial modulator of microbiota (Borre et al., 2014; Sudo et al., 2014). The adult-like core microbiome develops through a progressive change in the composition and diversity of the microbiota. During the first weeks of life, the infant’s fecal microbiota changes from a low diversity of facultative anaerobic bacteria to a higher diversity of strictly anaerobic bacteria (Bäckhed et al., 2015, Ferretti et al., 2018; Palmer et al., 2007). Full colonization takes approximately three years of age (Koening et al., 2011; Weng & Walker, 2013).

The brain, unlike most organs, undergoes critical development steps during the postnatal period when significant morphological growth, cell differentiation, and functional acquisition occur (Borre et al., 2014). Synaptogenesis increases throughout infancy and synaptic density reaches its highest value at two years of age when the brain has about twice as many synapses than the adult brains (Herschkowitz et al., 1997; Stilles et al., 2010). Following this synaptogenesis phase, elimination of synapses is necessary for developmental remodelling, a refinement process that strengthens and consolidates neuronal circuits acquired during childhood's neurobehavioral development (Borre et al., 2010). Furthermore, neuron-glia interactions are being defined and play a critical role in networks' functional organization throughout postnatal life. However, the current understanding of these processes is limited to human post-mortem data and extrapolation of findings from other species. Current research using in vivo imaging of children provides information about the temporal evolution of the developmental processes and how they connect to the evolving behaviours (Stilles et al., 2010). Information is also provided by magnetic resonance imaging – commonly used in children – which allows monitoring of the changes in tissue chemistry thought time, and

subsequent correlation with neural and glial proliferation and migration gives insight into the timing and anatomical distribution of the neurodevelopmental events (Barkovich, 2000; Barkovich, 2005). Finally, in vivo imaging studies are required to gain more profound knowledge regarding the dynamic interactions within the human brain tissue with a molecular and microstructural view (Stilles et al., 2010).

1.1.2 Gut-Brain Axis bidirectional communication with the brain

Over the last decades, the gut-brain axis communication's bidirectional nature has been elucidated, in which the microbiota plays a significant role (Cryan & Dinan, 2012; Evrensel & Ceylan, 2017; Hoban et al., 2016). The microbiota carries out its actions (protective, metabolic and trophic) by influencing immunologic, endocrine and neural pathways. These three psychoneuroimmunologic (PNI) pathways allow the microbiota to influence brain functions and neurodevelopment (Desbonnet et al., 2014; Heijtz et al., 2011; Hoban et al., 2018; Sudo et al., 2004) via mediation by cytokines, the hypothalamic-pituitary-adrenal axis (HPA) and the vagus nerve (Grenham et al., 2011, Yang et al., 2016).

Immune pathway

The immune system activity is essential in various brain processes (Dantzer, 2018; Morimoto & Nakajima, 2019; Dantzer & Wollman, 2003). As a result, the variation in microbiota and the resulting modulations in the immune system actions forms a potent pathway in microbiota-gut-brain-axis communication. The gut microbiome (the microorganism and their collective genetic makeup) contributes to the host's systemic inflammatory milieu by low-level leakage of bacteria and their component across the luminal intestinal interface (Duerkop et al., 2009). Throughout early life, the gut microbiota is an essential component of both innate and adaptive immune system development (Olszak et al., 2012). In the infant, early life dysbiosis of the microbiome may impact the developing immune system with a higher risk of developing an inflammatory disease (Yang et al., 2016). Moreover, gut microbes and/or their secretions can affect both the pro-inflammatory and the anti-inflammatory mediators essential for general immune homeostasis (Wu & Wu., 2012; Lee et al., 2011). For instance, levels of pro- and anti-inflammatory immunes mediators (e.g., cytokines) seem to alter behaviour and neurochemistry in rodents (Miller et al., 2013). In adults, an excessive presence of pro-inflammatory species (e.g., gram-negative *Enterobacteriaceae*) causes chronic low-grade inflammation, whereas the dominance of anti-inflammatory species (e.g., *Lactobacillus*) maintains a milieu inhospitable to pro-

inflammatory species (Adams & Hall, 1988; Haarman & Knol, 2006). Hence, the relation between the gut microbiota and the immune system activity is bidirectional and complex (Maynard et al., 2012).

Endocrine pathway

The microbiota's makeup affects the hypothalamic-pituitary-adrenal (HPA) axis, the primary stress regulation system (Sudo et al., 2004). The HPA axis is the central neuroendocrine system controlling cortisol output, a key regulator of glucose, protein, and lipid metabolism and immune responses (Bellavance & Rivest, 2014). Modulation of HPA axis function by the gut microbiota directly influences cortisol secretions and the normal development of the stress response (Sudo et al., 2004; Sudo et al., 2014) and indirectly influences activation of the HPA axis by pro-inflammatory cytokines (Ekenkov & Chrousos, 2002). During early life, HPA axis development and functioning are intertwined with specific bacteria colonizing the gut, such as *Bifidobacteria* (O'Mahony et al., 2017; Sudo et al., 2004). This microbiota-endocrine interplay is essential because various disorders of the microbiota-gut-brain axis (e.g., ADHD) might be associated with dysregulation of the HPA axis (Isaksson et al., 2012).

Neural pathway

The microbiota and/or its metabolic products may affect the nervous system innervating the gastrointestinal tract, including the autonomic nervous system (Wehrwein et al., 2016) and the enteric nervous system. The vagus nerve is a significant component of the autonomic nervous system, specifically the parasympathetic nervous system, and consists of afferent (80%) and efferent neurons. Also, it plays an essential role in microbiota-gut-brain communication. Hormonal, chemical, mechanical and nociceptive information from microbiota signals stimulates vagus nerve afferent endings and reaches the central nervous system (Berthoud et al., 2004; Egerod et al., 2018; Yang et al., 2016). Therefore, the output from the afferent endings may affect behavioural and efferent neuronal activity. However, in the early postnatal life, the vagus is not fully functional as it is only partially myelinated (Porges and Furman, 2011). Thus, signals from the gut microbiota may be altered during vagus nerve myelination in early postnatal life. Moreover, activation of afferent outputs is thought to be responsible for our "gut-feelings". Similarly, pro-inflammatory cytokines provide information regarding systemic inflammation (Yang et al., 2016). The enteric nervous system, mesh-like system within the gastrointestinal tract, works in conjunction with the

autonomic nervous system to ensure proper functioning and regulation of gut functions (e.g., motility). The enteric nervous system is sensitive to microbial metabolites (Mao et al., 2013; Hyland and Cryan, 2016), and its normal function is essential for microbiota stability (Rolig et al., 2017). Additionally, gut serotonin signalling potentially mediate gut microbiota influence in enteric nervous system maturation (De Vadder et al., 2018).

Basal metabolites

In addition to the three PNI pathways, it has long been known that the gut microbiota produces metabolites that may also act as neurotransmitters (Yano et al., 2015). The majority of serotonin biosynthesis occurs in the intestinal tract via enteroendocrine cells present in the epithelium and is promoted by gut microbiota (Yano et al., 2015). Similarly, biosynthesis of gamma-ammino-butyric acid (GABA) and dopamine (DA) is also predominantly done in the intestines by the gut microbiota (Dinan et al., 2015; O'Mahony et al., 2017). However, it is unknown how the neurotransmitters and the microbiota interact, to what extent these neurotransmitters are systemically absorbed and cross the blood-brain barrier, and what are the significant effects on neurodevelopment (Jameson et al., 2020;).

Apart from neurotransmitters, the gut microbiota is known to metabolize and utilize amino acids and short-chain fatty acids (SCFA). Amino acids play a role in neurotransmitter synthesis (Choi et al., 2009; Gao, Mu, Farzi & Zhu, 2020), whereas SCFAs are fermentation products of complex carbohydrates (Dalile et al., 2019), modulating immunity, metabolism and epigenetic programming (Krautkramer et al., 2016). Microbiota interventions may affect the central nervous system via alteration in amino acid metabolism (Olson et al., 2018) or SCFA levels (Bishehsari et al., 2018; Goswami et al., 2018).

1.1.3 Studying the relationship between the gut microbiota and the brain structural and functional development

Studying the microbiota

To understand the role of the gut microbiota on human health, researchers such as the Human Microbiome Project, aimed to characterize the microbiota composition and diversity. Traditionally, gut microbiota's characterization has been performed with selective in vitro culture methods, however with a limited range of identification. More recently, culture-independent methods have been used, including fluorescent in situ hybridization, gel electrophoresis, 16S ribosomal RNA cloning, sequencing, and quantitative polymerase chain

reaction (PCR) (Brooks, 2013). These more contemporary molecular methods are widely used in studies of the microbiota.

All bacteria have a highly conserved 16S ribosomal RNA gene containing hypervariable regions (Morgan & Huttenhower, 2014). Amplicon sequencing, also known as 16S rRNA sequencing, allows the amplification of these hypervariable regions, with a PCR followed by sequencing, and thus, obtention of an estimate of the proportions of detected bacteria in the sample (Morgan & Huttenhower, 2014; Cryan et al., 2019). However, despite being widely used for its low cost, the 16S rRNA sequencing is prone to limitations. For instance, PCR primer, amplification and accuracy biases are most notable because the 16S gene is subject to copy number variation. Furthermore, 16S rRNA sequencing limits reliable taxonomic annotation to the genus level and provides no information the genome or function of the detected bacteria (Morgan & Huttenhower, 2014). Shotgun metagenome (whole DNA) or metatranscriptome (whole RNA) sequencing yields information on the microbiota's functional capacity or functions at the species, gene or gene expression level. Furthermore, shotgun sequencing provides abundance estimates for bacteria, archaea, fungi and viruses, whereas 16S rRNA sequencing is limited to abundance estimates for bacteria (Morgan & Huttenhower, 2014). The late narrowing in cost difference between 16S rRNA sequencing and shotgun sequencing resulted in two-stage experiments integrating the two methodologies (Morgan & Huttenhower, 2014; Tickle et al., 2013).

Molecular methods have been critical in studying microbial communities in health and disease. Indeed, the characteristics and function of gut microbiota have been associated or implicated with, for example, the development of metabolic disease (Koh and Bäckhed, 2020) or plays a mechanistic role in the pathogenesis of neurological diseases such as multiple sclerosis (Cekanaviciute et al., 2017) and Parkinson's disease (Sampson et al., 2016; Baizabal-Carvallo and Alonso-Juarez, 2020). Hence, information about the gut microbiota's role in the underlying mechanisms may open doors for new interventions.

Studying the Microbiota-Gut-Brain Axis in animal models

In recent decades, the interplay between brain functioning, behaviour and gut microbiota has begun to be elucidated (Cryan et al., 2019; Hooks et al., 2018). In vivo models, especially rodents, under germ-free (GF) conditions are an essential starting point in understanding the gut microbiota's effects on brain functions and behaviour (Evrensel & Ceylan, 2017). Indeed, GF models demonstrated that the absence of microbiota in early life

influences multiple aspects of neurodevelopment (Borre et al., 2014) and leads to altered behaviour (Heijtz et al., 2011; Neufeld et al., 2011), social functioning (Desbonnet et al., 2014; Cryan & Dinan, 2015) or stress responsivity (Sudo et al., 2004). Similarly, GF conditioning leads to modification in levels of neurochemicals, such as neurotransmitters and neurotrophic factors (Bercik et al., 2011; Mayer et al., 2014; Sudo et al., 2004), and an increased blood-brain barrier permeability (Braniste et al., 2014). The GF rodent models do not have clinical correspondence and have limited translatability to humans; therefore, observed brain and behaviour differences are restricted to GF conditions. Additionally, dietary interventions and antibiotic and probiotics treatments can alter the gut microbiota, leading to behavioural and neurochemical changes (Cryan et al., 2019; Sudo et al., 2004). Intriguingly, early life microbiota is essential for behavioural outcomes, as changes induced by microbiota alterations are reversible by early-life colonization (Heijtz et al., 2011) but not necessarily during adulthood (Neufeld et al., 2011). Moreover, the changes caused by non-functional microbiota during early life have may been sex specific. On one hand, the neurochemical and endocrine effects under GF conditioning are mainly noticeable in male rodents (Cryan & O'Mahony, 2011). Contrastingly, female mice showed a reduction in anxiety behaviour and increased brain-derived neurotrophic factor (BDNF) (Neufeld et al., 2011). Social development is also impacted by gender variability, particularly under GF conditions where mice exhibited autistic traits predominantly observed in males (Desbonnet et al., 2014).

Studying the brain structure and function

The emergence of brain imaging techniques in the late 20th century (1980), such as positron emission tomography (PET), magnetic resonance imaging (MRI), and diffusion tensor imaging (DTI), led to the conclusion that there are interactions between the gut and the brain in humans. Brain imaging offers an ideal method for observing the influence of gut microbiota on the brain *in vivo*. Previous studies complementized their experimental imaging designs with neuropsychological measures or cognitive testing to evaluate the interactions between the microbiota composition and the brain or behaviour in health and neuropsychiatric disorders (Cryan et al., 2019).

MRI techniques (e.g., structural MRI and functional MRI) are widely used because of their non-invasive property and provide information regarding the brain's structural, function, and metabolic status (Liu et al., 2019). Structural MRI typically assesses gray matter structures in terms of volume and thickness, whereas functional MRI assesses cerebral

structures in terms of functional changes using indirect measure of regional blood flow (Liu et al., 2019). Preclinical studies brought evidence of structural, region-specific changes in GF mice's brain (i.e., cortex and hippocampus) in association with gut microbiota products (e.g., BDNF) (Sudo et al., 2004). Also, clinical evidence showed sex-dependent variation in cortical thickness of the anterior insular cortex and gray matter volume of irritable bowel syndrome patients (Weaver et al., 2016). Functional MRI, specifically blood-oxygenation level-dependent signal (BOLD), is commonly used for studying the neural basis of cognition (Liu et al., 2019). This technique generally relies on patient participation because it is tasks-based to visualize cerebral responses for specific cognitive processes (Liu et al., 2019). However, resting-state BOLD functional MRI can be performed to measure spontaneous cerebral activity (Liu et al., 2019). Hence, MRI techniques permit the visualization of the gut-brain interactions (i.e., Sudo et al., 2004), when associated with concomitant evaluation or modification of the gut microbiota status, and obtention of measurements of the potential structural and/or functional changes associated with the microbiota.

1.2 Microbiota-Gut-Brain Axis relationship with deep brain structures

The extensive work on animal models provided essential information regarding the microbiota-gut-brain axis's principal mode of action. However, the literature that translate preclinical information to human populations, especially infants, is substantially scarce. Despite investigations regarding the microbiota-gut-brain axis association with neuropsychiatric or neurodevelopmental disorders, there is a critical need for further studies on healthy infant populations. Indeed, unravelling the relationship between the gut microbiota and the brain structure and function in early life would provide a critical understanding of the origins of the microbiota-gut-brain axis and its neurodevelopmental association.

1.2.1 Microbiota-Gut-Brain Axis bidirectional communication in clinical studies

The microbiota-gut-brain axis seems to be implicated in multiple neuropsychiatric disorders, especially in depression (Bastiaanssen et al., 2018; Ding et al., 2019). Indeed, some evidence demonstrated probiotics' ability to modulate clinical depressive symptoms, yet limited studies focus on depression (Ng et al., 2018; Kelly et al., 2016). Additionally, studies suggested a possible association between the gut microbiota and other neuropsychiatric disorders, such as bipolar disorder (Hu et al., 2019; Gondalia et al., 2019) or rodent models for schizophrenia (Zheng et al., 2019). Also, several studies investigated the implication of the

microbiota-gut-brain axis in the development of neurodevelopmental disorders, such as autism and attention-deficit hyperactivity disorder (ADHD) (Bundgaard-Nielsen et al., 2020). They demonstrate a clear difference in fecal microbiota composition between autistic patients and healthy controls, with autistic patients possessing a greater gut microbiota beta-diversity, but this variation has not been consistently reported in the literature (Bundgaard-Nielsen et al., 2020; Finegold et al., 2010). Similar findings were found for ADHD, as gut microbiota is seen as an important external factor linked to the development of ADHD (Bundgaard-Nielsen et al., 2020; Cenit et al., 2017). In all, these observational studies on neuropsychiatric and neurodevelopmental disorders suggest the presence of a lifelong communication between the microbiota, the gut and the brain in humans. However, further studies should focus on early life associations between the gut microbiota and brain structure and function.

1.2.2 Association between fecal microbiota and brain structure and function during infancy or childhood

Only recently, microbiota-gut-brain studies have reported associations between fecal microbiota and brain structural and functional differences during infancy or childhood (Gao et al., 2019; Carlson et al., 2018; Callaghan et al., 2019; Sordillo et al., 2019). Despite being limited, they provide foundational information regarding microbiota influence on brain structure and function in early life. A study by Carlson et al. (2018) provided initial evidence of associations between gut microbiota and cognitive performance during infancy. Their results show a correlation between higher alpha diversity and lower Mullen Early Learning Cognitive Composite (ELC) scores at two years of age. They also evaluated the correlation between alpha diversity and various cerebral volumes and found a negatively directed association between alpha diversity and volumes for the amygdala, the left precentral gyrus and the right angular gyrus. Another study by Gao et al. (2019), interested in the brain's functional connectivity during infancy, provided primary evidence that microbiota diversity was associated with functional connectivity of neural circuits essential in cognitive development. Specifically, they showed a strong association between alpha diversity and weaker functional connectivity for two networks (ie., the amygdala and thalamus; the anterior cingulate cortex and anterior insula) (Gao et al., 2019). Besides, microbiota composition in infants has been associated with functional reactivity in brain networks of fearful faces (Callaghan et al., 2019) and developmental ages and stages (Sordillo et al., 2019). Communication, personal, social, and motor developmental scores appear to associate with particular bacterial genus abundances (e.g., *Bacteroides* and motor scores) (Sordillo et al.,

2019). Similarly, different bacterial genus abundances associate positively with different cerebral structures involved in the fear processing network (Callaghan et al., 2019). Moreover, studies showed an association between microbial diversity and cognitive and emotional functioning (Aatsinki et al., 2019; Carlson et al., 2018, Christian et al., 2015). Temperament, the innate part of character affecting our moods and behavior (i.e., self-regulation and emotional reactivity) (Rothbart, 2011), has been associated with microbiota alpha and beta diversity, specifically regarding fear reactivity and negative emotionality (Christian et al., 2015; Aatsinki et al., 2019).

In all, the emerging studies focusing on the interplay between the gut microbiota and brain structural (e.g., volumes) and functional (e.g., cognition, functional networks) development in early life show promising results. Indeed, they expend the current information obtained from preclinical studies as well as neurodevelopmental studies. Furthermore, specific structures, such as the amygdala, appear to be significantly influenced by the microbiota-gut-brain axis in health and neurodevelopmental studies. The recent studies by Gao et al. (2019) and Carlson et al. (2018) showed an association between the microbiota characteristics and the amygdala volume and functional connectivity. Hence, further research to expand our knowledge of microbiota-gut-brain influence on brain structures critical for neurodevelopment and healthy functioning, such as the striatum, is necessary.

1.2.3 The striatum is a critical component of the motor and cognitive systems

The basal ganglia regroup nuclei are located in the most central regions of the brain. They are composed of two principal structures, the striatum and the pallidum—each of these structures regrouping multiple nuclei. The striatum is composed ventrally by the caudate nucleus and the putamen, and dorsally, the nucleus accumbens. The pallidum regroups the globus pallidus (external and internal) and the substantia nigra. This organization of the basal ganglia structures rests on the functional subdivision of each nucleus. Indeed, the striatum and the pallida are the basal ganglia's input and output zones, respectively. The basal ganglia are involved in movement modulation (Mink & Thach, 1993) and non-motor functions implicating prefrontal and limbic pathways necessary for cognitive and emotional processing (Provost, Hangaru et Monchi, 2015).

The striatum is the principal component of the basal ganglia, which receives afferent information from the cerebral cortex and relays it to different structures in order to feedback the processed information to the cortex (Zorumski & Rubin, 2011). It is composed of three

nuclei, the caudate nucleus, the putamen and the nucleus accumbens. Moreover, the striatum is essential for both motor and non-motor functions of the basal ganglia. Movement modulation principally involves medium spiny neurons (MSNs) of the caudate nuclei and putamina, together with cortical neurons. Additionally, the corticostriatal pathway integrates afferent information with a functional and topographical organization (Goldman-Rakic & Selemon, 1990; Mink & Thach, 1993). Indeed, this highly conservative functional and topographical organization is also reflected in the striatum's microstructure, such as the nature of its synapses (e.g., glutamatergic, serotonergic, GABAergic and dopaminergic) (Goldman-Rakic & Selemon, 1990). More recently, along with the generalization of imaging techniques in experimental designs, the striatum's implication in the non-motor brain process has been unravelled (Provost, Hangaru and Monchi, 2015). Together with the frontal cortex and limbic structures, such as the dorsal prefrontal cortex, the anterior cingulate and the orbitofrontal cortex, the striatum plays a crucial role in cognitive and emotional mechanisms (Provost, Hangaru et Monchi, 2015). Hence, it has been suggested that disruptions in the functions of the striatum might be involved in the development of neurological and neuropsychological disorders, such as Parkinson's disease (Hanganu, 2015; Zhai et al., 2018), schizophrenia (McCutcheon et al., 2019), or depression (Pitenger & Duman, 2008; Liu et al., 2017). Furthermore, the striatum's involvement in these disorders' pathophysiology may be related to the variation of neurotransmitters expression (dopamine, glutamate, GABA, or serotonin), critical to these disorders in the striatum (The Human Memory, 2019).

During prenatal development, the striatum emerges from the ventral subdivision of the forming telencephalon (Onorati et al., 2014). Furthermore, the striatum's differentiation occurs in a very narrow temporal window (6-11 weeks of development) and is dependent on specific transcriptional dynamics involving about 90 genes (Onorati et al., 2014). Based on in vitro evidence, the striatum neurons are functional, showing spontaneous activity capabilities and specific ionic and synaptic conductance in response to neurotransmitter modulation (Onorati et al., 2014). Throughout infancy, brain growth is important to reach about 80-90% of its adult volume (Heijtz, 2016). Indeed, a net increase of gray matter volumes, including the striatum, has been observed and may correlate with crucial cognitive development (Knickmeyer et al., 2008). Most specifically, the caudate nucleus volume increases significantly between the age of one and two years, which may correlate with motor development, as it is a critical structure of movement modulation (Herrero et al., 2002; Knickmeyer et al., 2008). Excessive growth of the caudate nucleus has been associated with

the repetitive behaviours observed in autism (Qiu et al., 2014). However, there is limited literature regarding the development of the striatum in humans.

2 Aims of the thesis

Central aspects of gut-brain axis research inspect microbiota influences on cognition, mood, neurodevelopmental and psychiatric disorders (Hooks, Konsman, and O'Malley, 2018; Ding et al., 2019). Furthermore, it has been shown that deep brain structures are critical components of both gut-brain and neurodevelopment research (Zorumski & Rubin, 2011). Gut-brain axis studies extensively focused on gut microbiota influence the role and structure of the various deep brain component such as the amygdala both in health and pathology (Carlson et al., 2018; Gao et al., 2019; Callaghan et al., 2019; Srodillo et al., 2019). Despite studies of the brain's development (Gilmore et al., 2011; Knickmeyer et al., 2008; Provost, Hanganu, and Monchi, 2015), including the striatum, the relationship between the gut microbiota and the striatum is currently not of interest, although the crucial importance of the early years of life for neurodevelopment and gut colonization has been extensively reviewed (Borre et al., 2014; Jena et al., 2020; Kelsey et al., 2019). However, microbiota-gut-brain studies failed to understand the origins of the relationship between gut microbiota and brain functions in early life (Carlson et al., 2018). As of today, only a few studies have begun to develop our understanding of the gut-brain relationship in early life (Gao et al., 2019; Carlson et al., 2018; Callaghan et al., 2017; Sordillo et al., 2019). The study by Carlson et al. (2018) suggested an association between microbial diversity in the gut, cognitive development and various cerebral volumes. However, their results showed an absence of association at one or two years of age between the gut microbiota diversity and the caudate nucleus. Hence, to address the paucity of the current literature, further studies are needed to understand the microbiota-gut-brain axis concerning early life events.

This cross-sectional exploratory study will examine the origins of the gut-brain relationship, especially the interplay between the microbiota composition and diversity and the striatum's volumes. Specifically, the study will examine how the composition and diversity of infant fecal microbiota at the age of 2.5 months are associated with striatum volumes, particularly the caudate nuclei and putamina, at approximately one month and whether a sex-specific interaction is present among these associations.

We hypothesize an observation of associations (positive or negative) between the gut microbiota diversity and the striatum volumes as previously suggested for particular subcortical structure (i.e., amygdala) in prior literature (Carlson et al., 2018; Gao et al., 2019).

3 Methods

3.1 Participants

The study population (n=56; 24 boys and 32 girls) is drawn from the ongoing FinnBrain Birth Cohort study conducted in South-West Finland (www.finnbrain.fi, Karlsson et al., 2018), which has been approved by the Ethics Committee of the Hospital District of Southwest Finland. Criteria of infant's inclusion were usable fecal samples and structural magnetic resonance imaging data. Among the ~200 available infant scans with measures of striatum volumes, only 56 also provided fecal samples for microbiota evaluation. The FinnBrain research group aims to study the combined influences of genetic and environmental factors during early life on posterior child development and health outcomes. Recruitment of families lasted from December 2011 until April 2015 and was done during the first ultrasound appointment in maternity clinics. In the invited parents, 66% agreed to participate in the cohort study. A total number of 3837 children are taking part in the birth cohort study. All mothers provided written informed consent on behalf of their child. The details of the recruitment of cohort participants (both children and their parents) are detailed in a previous paper by Karlsson et al. (2018).

3.2 Covariates

Mothers provided background information through self-report questionnaires both prenatally and postnatally, as described in Aatsinki et al. (2019). Prenatal questionnaires were filled at gestational weeks (gwk) 14 and 34. Prenatal information included the level of mother's education (1: university education, 2: vocational school diploma, 3: secondary level education or equivalent diploma) and the maternal use of selective serotonin reuptake inhibitors or serotonin-norepinephrine reuptake inhibitors (SSRI/SNRI). Postnatal questionnaires provided information on infant antibiotic use and feeding mode in months. The breastfeeding variable was categorized as no breastfeeding, partial breastfeeding, exclusive breastfeeding, and ceased before 2.5 months of age, according to Stewart et al. (2018). Data on maternal pre-pregnancy body mass index (BMI; kg/m^2), infant's birth weight (g), height (cm), duration of gestation (weeks; preterm <37 gwk, term 37-41 gwk, post-term ≥ 41 gwk), antibiotics intake during the neonatal period, and the mode of delivery (caesarian-section or vaginal) was collected from National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi) (Karlsson et al., 2018).

3.3 Acquisition of subcortical gray matter structures volumes

3.3.1 MRI acquisition

The magnetic resonance images (MRI) were obtained with a Siemens Megnetom Verio 3T scanner (Siemens Medical Solutions, Erlangen, Germany). The acquisition protocol has been described in detail by Lehtola et al. (2019). The subjects underwent a 40-minute protocol during normal sleep. This protocol included two scanning sequences: (1) an axial PD-T2-TSE (Dual-Echo Turbo Spin Echo with repetition time [TR]: 12 070 ms, effective echo times [TE]: 13 ms and 102 ms) and (2) a sagittal 3D-T1 MPRAGE (Magnetization Prepared Rapid Acquisition Gradient Echo with TR: 1900 ms, TE: 3.26, inversion time: 900 ms). All images obtained were medically assessed by a neuroradiologist for incidental findings.

3.3.2 Assessment of structure volumes

The left and right caudate nuclei and putamina volumes were assessed via a template library and a label-fusion-based method. The procedure for assessing volumes has been previously described in detail by Acosta et al. (2020). Simply, it has three steps. First, the construction of a template library-specific to the population at hand and the manual labelling of all the subcortical structures of interest. Second, the construction of a library of wrapped versions of the labelled template allows a representation of the morphological variations. Third and last, the individual labelling of each subject's scans by performing a label-fusion-based method and calculating deep gray matter structure volumes. A label-fusion method is used for individual segmentation, and the approach uses a population-specific template library for the structure of interest (i.e., caudate nucleus and putamen). The method used, based on label-fusion, is described in Acosta et al. (2020) and emanates from complementary works of Coupé et al. (2011), Weier et al. (2014), and Lewis et al. (2019) (Figure 1).

The mean and standard deviation for the calculated volumes of the left and right striatum (caudate nuclei and putamina) for the whole sample (N=56) and, independently, for girls (N = 32) and boys (N=24) separately, are recorded in Table 1.

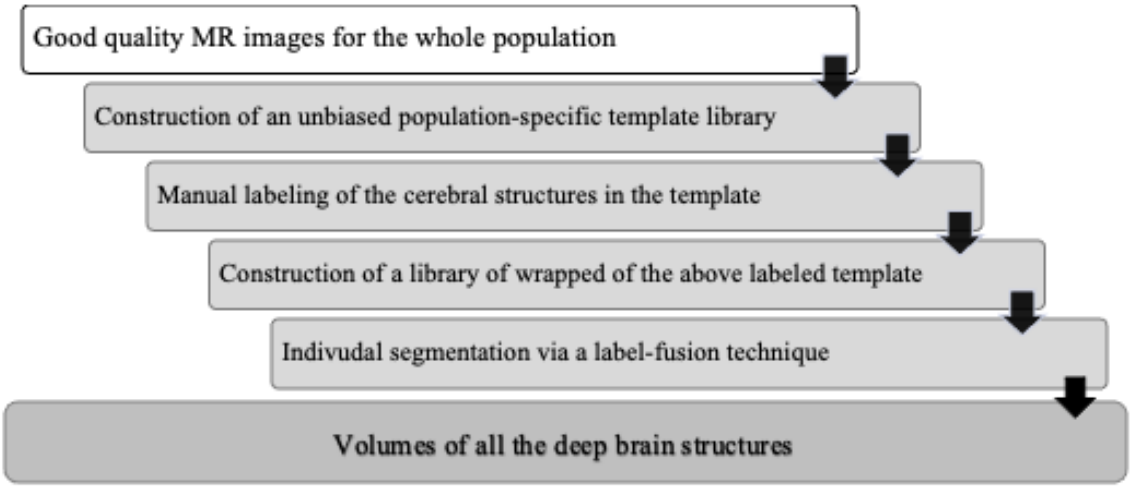

Figure 1. Assessment of the structure volumes protocol simplified from Acosta et al. (2020).

3.4 Acquisition of intestinal microbiota composition and diversity

Gut-microbiota characterization is complicated as the microbiota contains 100 times the human genome's genes (Eckburg et al., 2005). However, it is facilitated by rapid sequencing techniques in combination with bioinformatics. Once the fecal samples (gut microbiota) have been collected, they undergo a DNA extraction protocol followed by a 16S ribosomal RNA (rRNA) gene V4 region sequencing protocol. The 16S gene is composed of 9 hypervariable regions (V1-V9). The V4 region is commonly used for its size (~254 bp) and its low divergence in length (www.illumina.com, Application Note: Sequencing).

16S rRNA sequencing is a well-established method as a measure of microbial community structure and diversity. The 16S gene is universal across bacteria and archaea, thus making it an extensively used marker. The 16S sequences are phylogenetically informative allowing extrapolation of the microbial taxa. Although very common for comparison of samples phylogeny and taxonomy, 16s rRNA sequencing is limited. Indeed, microorganisms often have multiple copies of the 16S gene in their genome. Hence, the observed fluctuation in the 16S gene during sequencing can be explained both by variation in genomic 16S copy number and in the abundance of microorganisms (Kembel, Wu, Eisen, & Green, 2012).

3.4.1 Fecal sampling and 16S sequencing

The infant's fecal sample data was drawn from the FinnBrain study. Fecal sampling is an ideal collection method as it is non-invasive and easily performed at home for a target population of infants. Fecal samples were collected at approximately 2.5 months of age (age

mean = 10.4 weeks, SD = 1.8) at home by the parents as previously described in more detail by Aatsinki et al. (2019). Parents were instructed orally and in writing to collect a stool sample into a sterile collection tube, marked with the sampling's date and time. The sample tube was immediately stored either refrigerated or frozen until delivered with coolers to the study rapidly after the collection. Samples delivered to the study center within 48 hours after sample collection were homogenized by gentle mixing, weighed into aliquots, and frozen at -75°C until DNA extraction.

Bacterial DNA was extracted from 100 mg sample aliquots with GXT Stool Extraction Kit VER 2.0 (Hain Lifescience GmbH, Nehren, Germany). The extraction was otherwise performed according to the manufacturer's instructions. However, sample vortexing was replaced by homogenization with MOBIO PowerLyzer 24 Bench Top Bead-Based Homogenizer in 0.1mm glass bead tubes (MO BIO Laboratories, Inc., Carlsbad, CA, USA) at 1000 rpm for 3 minutes to induce cell lysis. The DNA concentrations were measured with Qubit dsDNA HS Assay kit and Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNAs were stored at -75°C. To analyze the fecal microbiota profiles, variable region V4 of the bacterial 16S rRNA gene was amplified with custom-designed dual-indexed primers and sequenced with Illumina MiSeq system as previously described by Rintala et al. (2018).

3.4.2 Obtention of Amplicon Sequence Variants (ASVs) and Shannon index of diversity

The raw sequences from the 16S rRNA gene sequencing were preprocessed with DADA2-pipeline (version 1.14) to infer exact amplicon sequence variants (ASVs) (Callahan et al., 2016). The reads were truncated to length 225 and reads with more than two expected errors were discarded (max EE = 2). Genome taxonomy database (GTDB, release 86) (Parks et al. 2020) and RDP Naive Bayesian Classifier algorithm (Wang et al. 2007) were used for the taxonomic assignments of the ASVs. The phylogenetic tree was constructed with package *phangorn* (Schliep 2011). The resulting sample had 14k-255k (mean = 83k, SD = 57k) reads per sample. The ASVs are used to determine the beta diversity and the differential abundance of the sample's microbiota.

Alpha diversity Shannon Index was determined with *microbiome* R package, on R software 3.5.0 (McMurdie and Holmes, 2013). Shannon Index was measured to present the

microbiota community's species diversity, as it a commonly used index in microbial studies (Shen, Zhang, Zhu, Zhang, & He, 2008).

3.5 Statistical analysis

The statistical analyses were performed with R 4.0.1. software (R Core Team, 2020). The R package *tidyverse* has been added to base R packages because it includes a set of functions, data, and documentation, necessary for the analysis (Wickham & Grolemund, 2016). The dependent variables were the right and left striatum volumes, i.e., caudate nuclei and putamina. The independent variables regrouped the gut microbiota parameters alpha and beta diversities. Covariate variables were chosen from theoretical assumptions formulated in existing literature (Pulli et al., 2018; Carlson et al., 2018; Gao et al., 2019). Covariates were assumed to be associated with either the independent or dependent variables – thus, those included in the analysis were sex, delivery mode, gestational age (at birth), age at fecal sampling, age at scan from birth, feeding mode (at the age of fecal sample) and birth weight. Antibiotic treatment was excluded from covariates, despite inclusion in previous literature, as it was poorly reported in the questionnaires and thus, was potentially relevant for only five infants. Furthermore, the significance level (alpha) was defined at 0.05 level. Shapiro-Wilk test was used to test the normality of the distribution for all the variables. Shannon Index (gut microbiota alpha diversity) and the striatum volumes were normally distributed (Table 1).

Table 1. Shapiro-Wilk test results for the independent and dependent variables. For statistical significance p-value <0.05.

Variable		Shapiro-Wilk test W	p-value
Shannon Index		0.98	0.57
Striatum volumes	Left Caudate Nucleus	0.97	0.24
	Right Caudate Nucleus	0.98	0.35
	Left Putamen	0.99	0.87
	Right Putamen	0.99	0.95

3.5.1 Associations between alpha diversity and striatum volumes, and sex differences exploration

The exploration of potential associations between the striatum volumes and alpha diversity (Shannon Index) was performed in R software, starting with a Spearman correlation coefficient test. Additionally, linear regression models were run for associations between the alpha diversity and the left and right caudate nuclei and putamina, with adjustments for the defined covariate variables.

Further, sex differences were explored first via stratified analyses of the correlation coefficient between sex and alpha diversity. Also, linear regression models were run to include an interaction term for sex (sex*diversity). The results obtained are reported on a scatter plot, done with the *ggplot* function of the *tidyverse* R package.

3.5.2 Evaluation of sample homogeneity and dissimilarity by calculation of microbiota beta diversity and dispersion effect size

To explore how the fecal microbial communities may differ between each infant, we measured the beta diversity and the beta dispersion of the sample. First, visualization of the data similarities (or dissimilarities) was obtained by performing a Principal Coordinates Analysis (PCoA) (Zuur, Leno & Smith, 2007; Gower, 1966, www.sequentix.de). The PCoA, with multi-dimensional scaling ordination of the ASVs, was performed with the *microbiome* and *phyloseq* packages in R (Lahti et al., 2012-2017) to visualize a summary of the infants' fecal beta diversity (genus level) with regards to the dependent, independent and covariate variables. Then, the beta diversity, testing whether the microbiota composition among groups, here individuals, is similar or not to one another (Goodrich et al., 2014), was investigated. The R package *vegan's* function *adonis*, which runs a permutational multivariate analysis of variance using OTU-wise distances (genus level) matrices (permutations = 999), was used to calculate the effect size (R2) of beta diversity for the independent and covariate variables. Finally, the beta dispersion, which tests the homogeneity of dispersion of composition among samples, here individuals, was investigated for all the covariate variables using the *betadisper* function's ANOVA method, part of the *vegan* R package. All the results, beta diversity and beta dispersion, are reported on PCoA plots, done with the *plot_ordination* function of the *phyloseq* R package.

3.5.3 Differential abundance of the gut microbiota

Differential abundance analyses, used to detect differences in taxonomic composition (https://www.biobam.com), were run for the left and right caudate nucleus and putamen, controlling for the defined covariate variables. The *DESeq2* R package was used to perform quantitative analyses of differential expression via shrinkage estimation, resulting in identifying bacterial signatures with a statistically significant association with striatal volumes (Love et al., 2014; Aatsinki et al., 2019).

4 Results

This study's main objective is to explore the origins in the infancy of the gut-brain interactions, specifically the relationship between the diversity of the intestinal microbiota and the brain volumes of the striatum. We expected the presence of associations (positive or negative) between the gut microbiota diversity and the striatum volumes as suggested for particular subcortical structure in previous literature (Carlson et al.,2018; Gao et al., 2019).

This exploratory study has been conducted by analyzing previously collected and processed data – magnetic resonance images for the striatum volumes and fecal samples for the gut microbiota diversity – by the FinnBrain research group. The analysis focused on alpha, beta diversities and the differential abundance of the intestinal microbiota. This exploratory research shows the absence of associations between the gut microbiota and the brain striatum volumes.

4.1 Participants' characteristics and main variables

The mean gestational age was 39.9 weeks. Most of the babies were born vaginally (80%). Mode of delivery data was missing for two individuals (1 boy and one girl). The majority of the infants were exclusively breastfed at the time of the fecal sample (77%). Only two boys were not breastfed at all. Also, there were no significant sex differences in breastfeeding patterns ($p = 0.53$) or birth mode ($p = 0.90$). There were no important variations in ages at fecal sampling (mean = 68 days) and at scan (mean = 25 days from conception) between boys and girls. (Table 2). The average difference between scan and fecal sampling is of 44 days (SD = 16).

Table 2. Study participants' clinical characteristics as mean (standard deviation, SD) or count (percentage, %). Gestational weeks = gwk, not available = NA.

Mean/Count (SD/%)		Overall	Boys	Girls
		n = 56	n = 24	n = 32
Gestational age, weeks		39.96 (1.16)	39.77 (1.15)	40.10 (1.16)
Gestational stage	Pre-term < 37 gwk	0 (0%)	0 (0%)	0 (0%)
	Full term < 40 gwk	30 (54%)	15 (63%)	15 (47%)
	Late term < 42 gwk	22 (39%)	8 (33%)	14 (44%)
	Post term ≥ 42 gwk	4 (7%)	1 (4%)	3 (9%)
Birth weight, g		3449.36 (385.29)	3448.542 (440.77)	3450 (343.89)
	NA	1 (18%)	0 (0%)	1 (31%)
Delivery mode, n	Vaginal	45 (80%)	19 (79%)	26 (81%)
	Cesarian (C)-section	9 (16%)	4 (17%)	5 (16%)
	NA	2 (4%)	1 (4%)	1 (3%)
Feeding mode, n	Exclusive breastfeeding	43 (77%)	17 (71%)	26 (81%)
	Partial breastfeeding	9 (16%)	4 (17%)	5 (16%)
	Cessation before 2.5 months age	1 (2%)	1 (4%)	0 (0%)
	No breastfeeding	1 (2%)	0 (0%)	1 (3%)
Age at fecal sampling, days		68 (14)	69 (15)	67 (14)
Age at scanning, days (from birth)		25 (7)	25 (8)	24 (5)

Mean volumes are for the caudate nuclei 1405.32 and putamina 1462.88. There is no difference in the right hemisphere caudate nucleus and putamen and left hemispheres structures volumes. Boys have higher striatum volumes overall than girls, however these results did not differ with statistical significance ($t= 0.88$, $p = 0.38$). Alpha diversity (Shannon Index, i.e., relative microbial abundance) of the infant's gut microbiota was higher in girls than boys ($t= 1.42$, $p = 0.16$) (Table 3).

Table 3. Main variables as means (standard deviation, SD)

Mean (SD)		Overall	Boys	Girls
		n = 56	n = 24	n = 32
Striatum volumes (mm^3)	Left Caudate Nucleus	1380.53 (140.34)	1387.28 (133.88)	1375.48 (146.91)
	Right Caudate Nucleus	1430.11 (145.07)	1440.67 (112.94)	1422.187 (166.51)
	Left Putamen	1471.06 (126.13)	1483.51 (95.20)	1461.718 (145.91)
	Right Putamen	1454.70 (115.33)	1473.51 (84.10)	1440.60 (133.69)
Alpha diversity (Shannon Index)		1.62 (0.52)	1.51 (0.48)	1.71 (0.55)

4.2 No association between alpha diversity and striatum volumes

Associations between gut microbiota alpha-diversity and striatum volumes were visually investigated on scatter plots with linear regression lines (Figure 2). Figure 2 displays the relationship between alpha diversity and striatum volumes separately for left and right caudate nuclei and putamina.

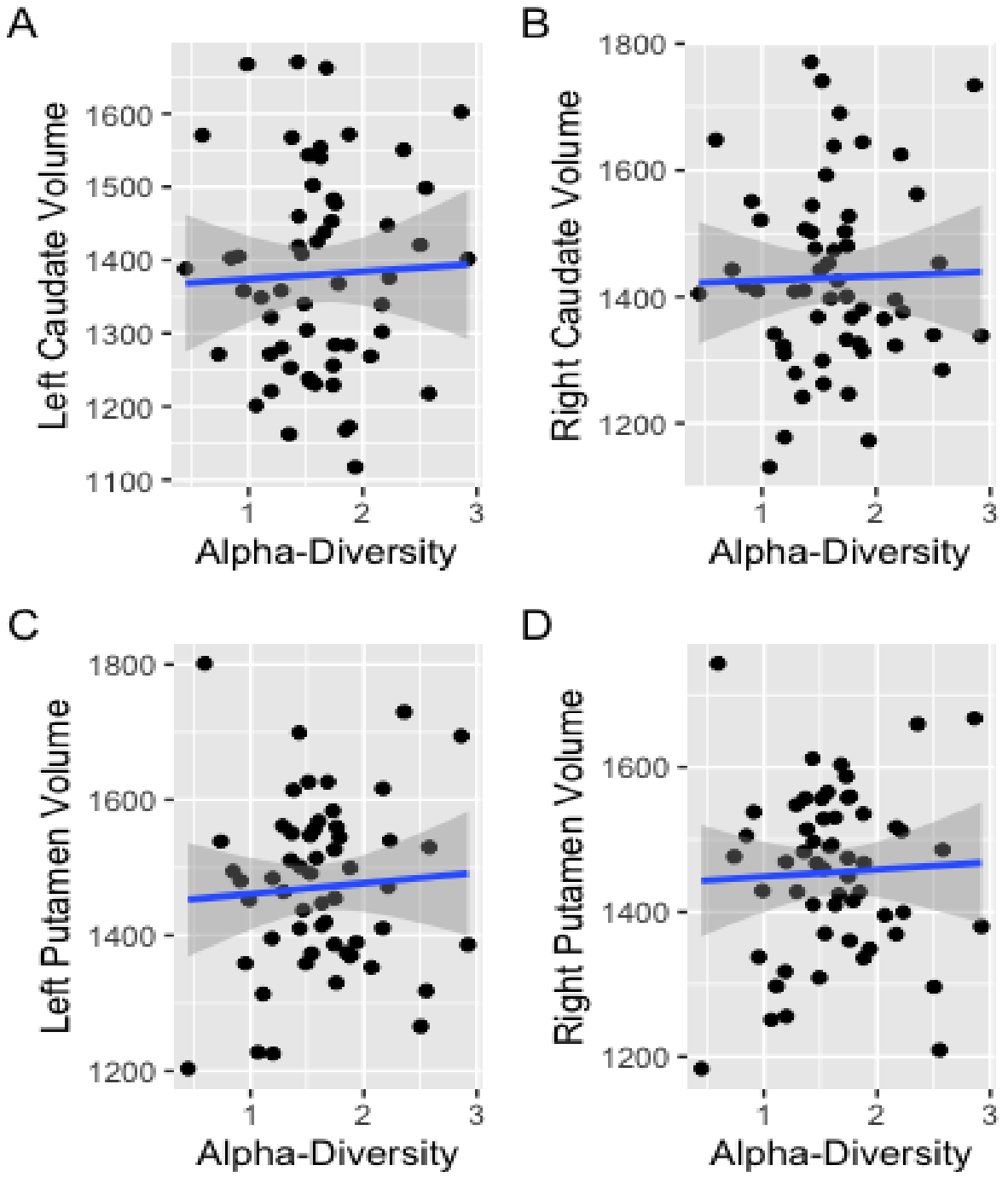

Figure 2. Scatter plots of brain volumes for A) left-caudate nucleus, B) right-caudate nucleus, C) left-putamen, D) right-putamen in function of the alpha-diversity of the gut microbiota. The slope of the blue regression line in each plot has been calculated by a linear regression model. Each point in the scatter plots represent an infant from the sample. Statistics performed by Spearman's r correlation test (A, r = 0.03, p = 0.75 / B, r = -0.04, p = 0.44/ C, r = 0.02, p = 0.68 / D, r = 0.01, p = 0.71).

Alpha diversity (mean Shannon Index = 1.62, SD = 0.52) did not correlate with any of the striatum volumes (left caudate nucleus (LC), right caudate nucleus (RC), left putamen (LP) and right putamen (RP)) in the unadjusted analyses (Spearman's correlation r for LC, r =

0.03, p = 0.75; RC, r = -0.04, p = 0.44; LP, r = 0.02, p = 0.68 and RP, r = 0.01, p = 0.71). When adjusted for mode of delivery, feeding mode, gestational age at birth, child sex, birth weight, age at fecal sample, age at scan and antibiotics treatment, no correlation was found with alpha diversity (LC, p = 0.75, R^2 = 0.09; RC, p = 0.44, R^2 =0.09; LP, p = 0.68, R^2 = 0.12 and RP, p = 0.71, R^2 = 0.11) (Table 4).

Table 4. Correlations between alpha diversity and striatum volumes, after controlling for covariates birth mode, feeding mode, gestational age at birth, sex, birth weight, age at fecal sample and age at the scan.

Striatum Volumes	R coef.	p-value	R^2
LC	0.03	0.75	0.09
RC	-0.04	0.44	0.09
LP	0.02	0.68	0.12
RP	0.01	0.71	0.11

4.2.1 Sex differences in alpha diversity associations with caudate nuclei and putamina

A stratified analysis for sex showed a slight difference in correlation between the alpha diversity and the striatum volumes, albeit not significant. Girls had a higher association between alpha diversity and the four striatal volumes (LC, r = 0.78, p = 0.44; RC, r = 0.77, p = 0.45; LP, r = 0.54, p = 0.60; RP, r = 0.53, p = 0.60) than boys (LC, r = 0.04, p = 0.97; RC, r = 0.65, p = 0.53; LP, r = -0.37, p = 0.72; RP, r = -0.08, p = 0.94). Also, the correlation evolved in opposite direction for both sex (Figure 3). However, after inclusion of an interaction term (diversity*sex) in the correlation analysis, the effect size revealed to be unsignificant, validating the previous results obtained during stratified analysis. Thus, no sex difference analyses were performed for beta diversity and differential abundance analyses.

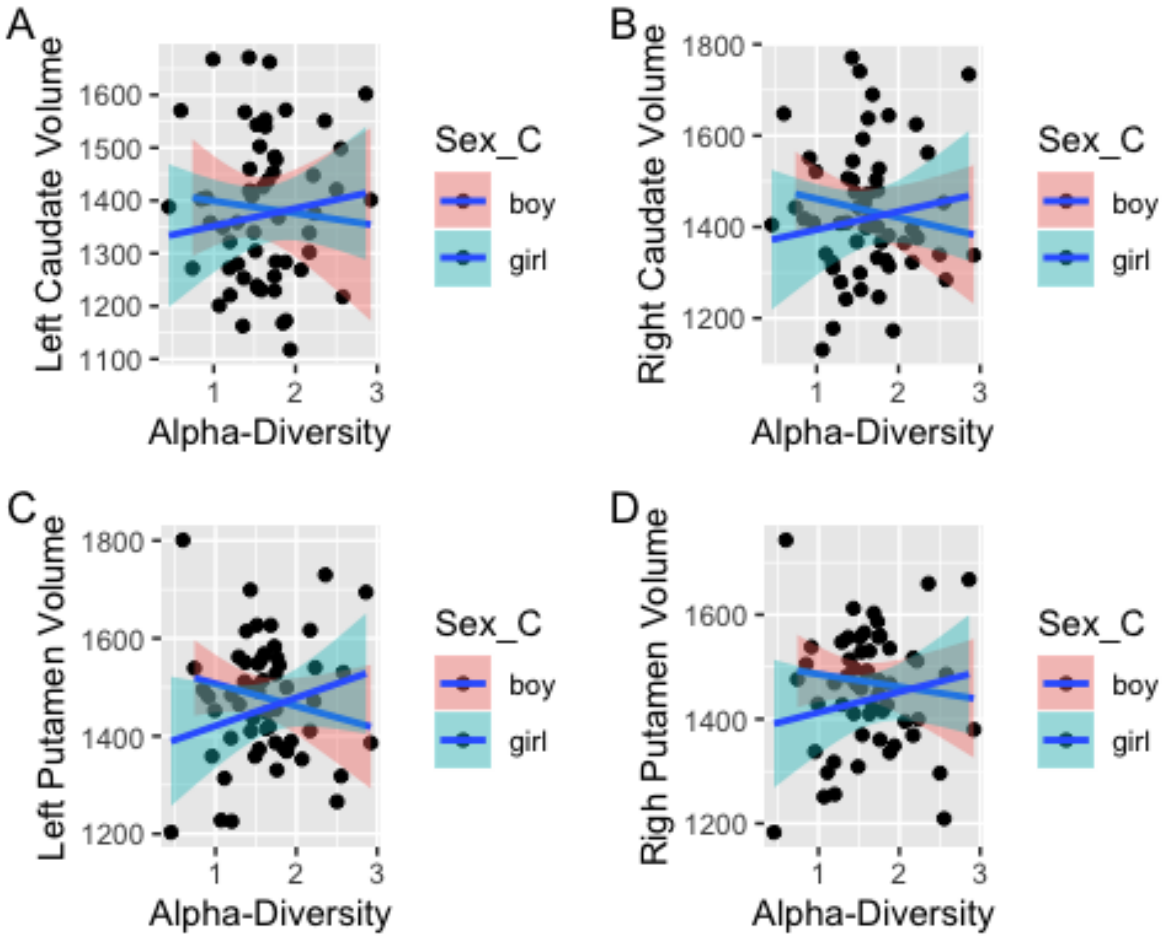

Figure 3. Scatter plots of brain volumes for A) left-caudate nucleus, B) right-caudate nucleus, C) left-putamen, D) right-putamen in function of the alpha-diversity of the gut microbiota with regards to sex. The slope of the regression lines in each plot has been calculated by a linear regression model. Each point in the scatter plots represent an infant from the sample. Statistics performed by Spearman's r correlation test for girls (LC, r = 0.78, p = 0.44; RC, r = 0.77, p = 0.45; LP, r = 0.54, p = 0.60; RP, r = 0.53, p = 0.60) and boys (LC, r = 0.04, p = 0.97; RC, r = 0.65, p = 0.53; LP, r = -0.37, p = 0.72; RP, r = -0.08, p = 0.94).

4.3 Absence of significant effect size for the beta diversity, despite similar beta distribution for covariates birth mode and sex.

Gut microbiota beta diversity was first examined visually with PCoA scatter plots (Figure 4). Figure 4 shows the relationships between beta diversity and striatum volumes variables separately for left and right caudate nuclei and putamina.

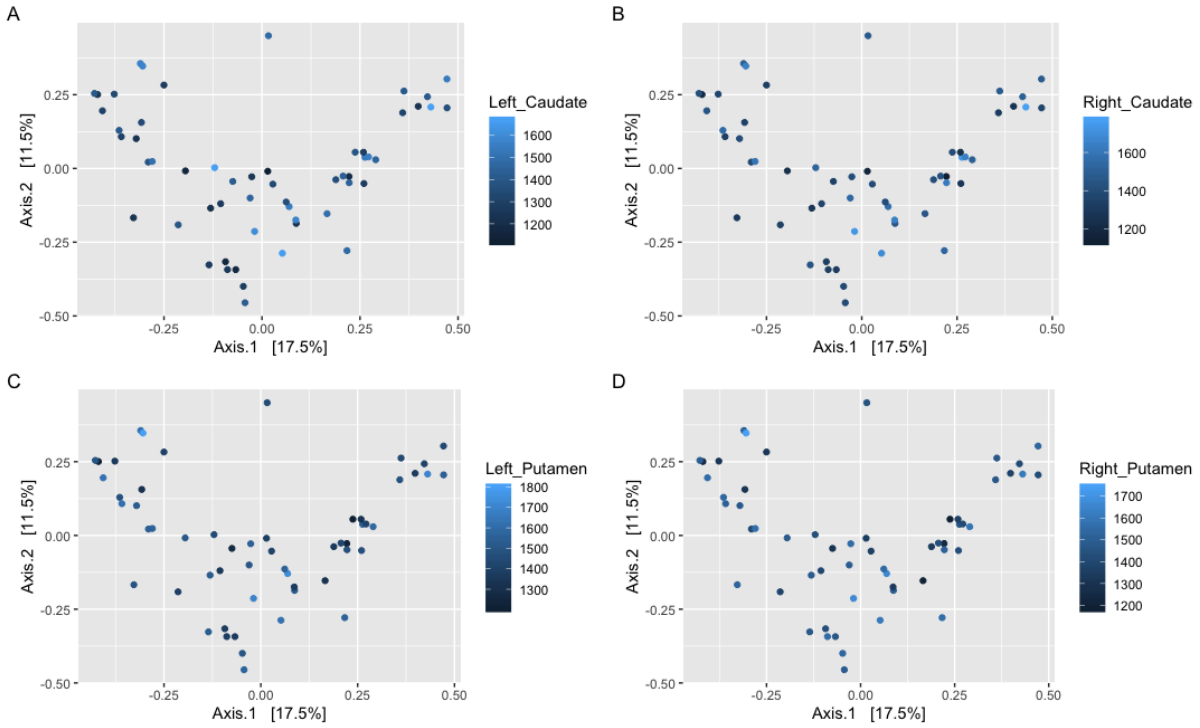

Figure 4. PCoA scatter plots for A) left-caudate nucleus, B) right-caudate nucleus, C) left-putamen, D) right-putamen, in the axis 1 and axis 2. The PCoA plots were obtained with the plot_ordination function of the phyloseq R package. Each point in the scatter plots represent an infant from the sample. Statistics performed by permanova test (A, R^2 = 0.01, p = 0.94 / B, R^2= 0.01, p = 0.57 / C, R^2 = 0.01, p = 0.61 / D, R^2 = 0.02, p = 0.35).

Beta diversities were insignificant for any of the striatum volumes (left caudate nucleus, right caudate nucleus, left putamen and right putamen) in the unadjusted analyses (LC, R^2 = 0.01, p = 0.90; RC, R^2 = 0.01, p = 0.82; LP, R^2 = 0.01, p = 0.87; RP, R^2 = 0.02, p = 0.51) (Table 5). When adjusted for birth mode, feeding mode, gestational age at birth, sex, birth weight, age at fecal sample, age at scan and antibiotics treatment, beta diversities were unsignificant with the striatum structure (LC, R^2 = 0.01, p = 0.93; RC, R^2 = -0.01, p = 0.57; LP, R^2 = 0.01, p = 0.61; RP, R^2 = 0.02, p = 0.35). However, small significance was found for beta diversity with the following covariate variables: child sex ($p < 0.05$) (Figure 5) and birth mode (<0.1) (Figure 6). Additionally, the beta dispersion, testing the similarity of variance in the composition among each individual, for all the covariate variables have been evaluated and showed a significant result for birth mode ($p < 0.01$) (Table 6).

Table 5. Correlations between beta diversity and striatum volumes after controlling for covariates birth mode, feeding mode, gestational age at birth, sex, birth weight, age at the fecal sample, and age at the scan. Significance of <0.05 and <0.1 for covariates sex and birth mode respectively for all striatum volumes.

Striatum Volumes	p-value	R^2
LC	0.94	0.01
RC	0.57	0.01
LP	0.61	0.01
RP	0.35	0.02

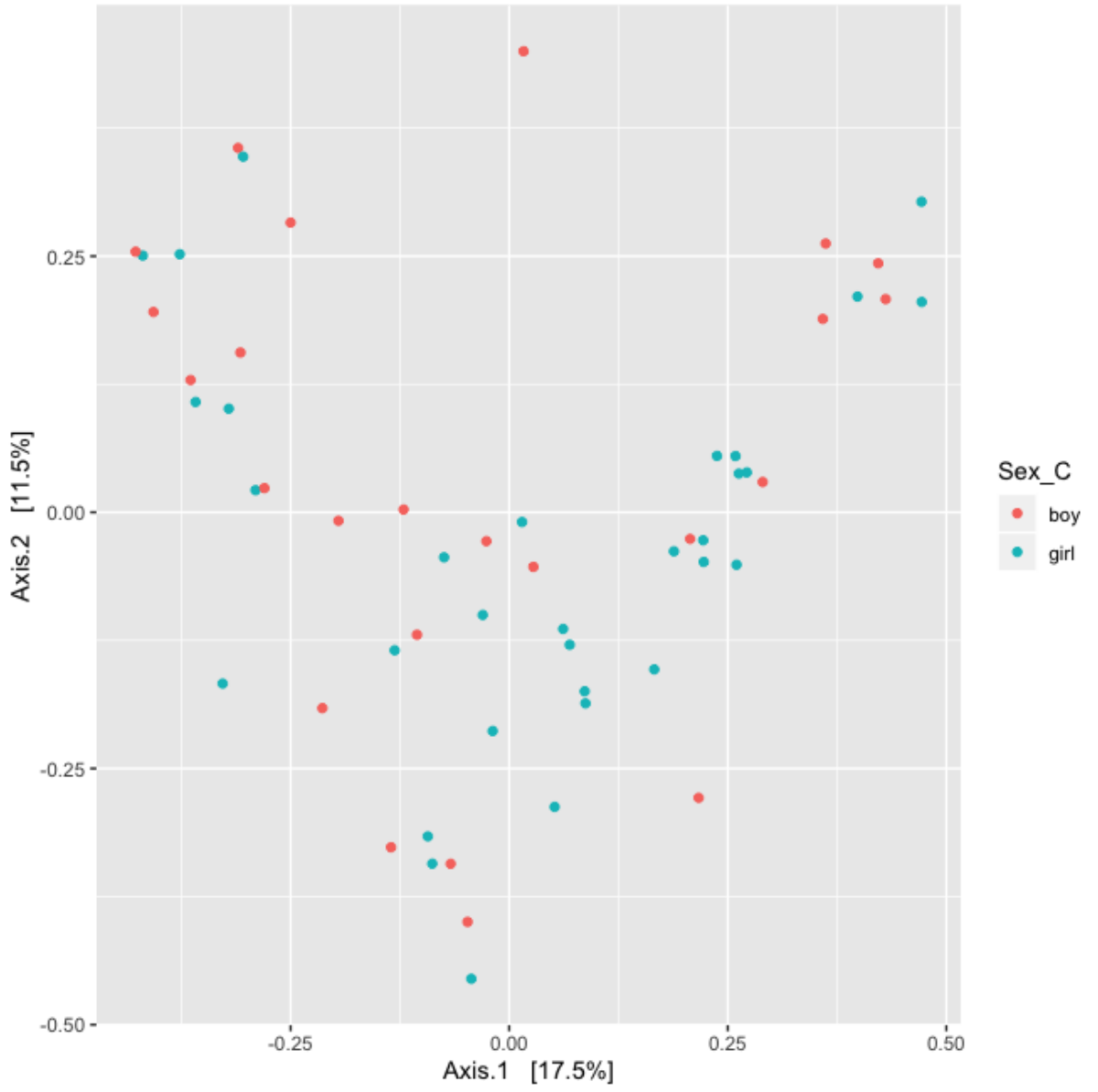

Figure 5. PCoA scatter plots for sex of the child ((F = 0.58, p = 0.45) in axis 1 and 2. The PCoA plots were obtained with the plot_ordination function of the phyloseq R package. Each point in the scatter plots represents an infant from the sample.

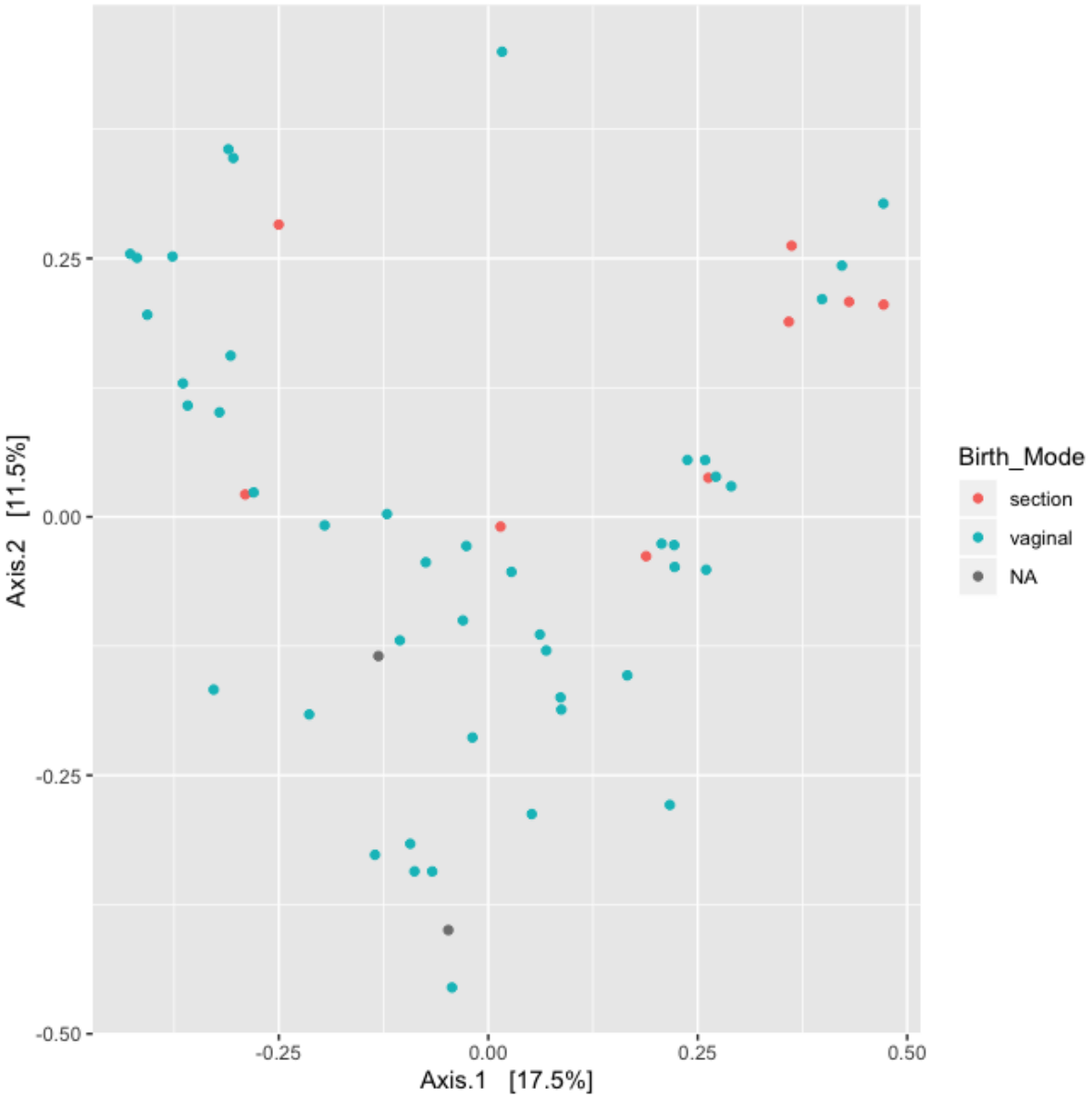

Figure 6. PCoA scatter plots for birth mode (F = 1.37, p = 0.25) in axis 1 and 2. The PCoA plots were obtained with the plot_ordination function of the phyloseq R package. Each point in the scatter plots represents an infant from the sample.

Table 6. Beta dispersion for covariates variables birth mode, feeding mode, gestational age at birth, sex, birth weight, age at the fecal sample, and age at the scan. *** p-value <0.

Striatum Volumes	F-value	p-value
Birth Mode	1.37	0.25
Gestational Age	1.13	0.38
Sex	0.58	0.45
Birth Weight	$2.61e^{+27}$	$< 2.2e^{-26}$ ***
Age at fecal sample	1.64	0.16
Age at scan	0.94	0.52

4.4 Absence of significant differential abundance at genus level in the sampled microbiota

Differential abundance of the microbiota at genus level was not correlated with any of the striatum volumes (left caudate nucleus, right caudate nucleus, left putamen and right putamen), when adjusted for birth mode, feeding mode, gestational age at birth, sex, birth weight, age at fecal sample, and age at scan (LC, mean = -0.04, p = 0.76; RC, mean = -0.13, p = 0.73; LP, mean = -0.12, p = 0.78; RP, mean = -.0.05, p = 0.75). The results are not graphically reported.

5 Discussion

Although gut-brain axis influence in neurodevelopment processes has been exposed in preclinical and clinical studies, we currently have a limited understanding of the initial interplay between gut microbiota and brain maturation during early life. In this study – explorative in nature – the aim was to shed light on the origins of the relationship between the infant gut microbiota diversity and deep brain structure evolution as previous literature is scarce. Despite modest effect size, an association between gut microbiota diversity, caudate nuclei, and putamina volumes was expected. Indeed, previous findings from Carlson et al. (2018) indicated a link between alpha diversity and various brain regions' volumes at two years of age. Previous literature also demonstrated associations with other structures such as the amygdala (Gao et al., 2019). Discordant with our hypothesis, our results did not attest of a significant association between the selected gut microbiota measures, intra-individual diversity and differences in overall composition, and the striatum volumes during infancy.

5.1 Absence of correlation between the gut microbiota characteristics and striatum volumes

As advanced in previous studies, gut microbiota may play a critical role in brain development in early infancy. Indeed, previous studies demonstrated that both the microbiota and brain critical but sensitive developmental windows were temporally coordinated (Borre et al., 2014). Furthermore, brain development is very vulnerable to internal and external environmental factors, such as gut microbiota and maternal prenatal health (Borre et al., 2014; Jena et al., 2020). Hence, developing our understanding of the co-development and bidirectional communication of gut and brain would allow early life interventions when gut microbiota modulation is possible (Christian, 2019). However, clinical microbiota-gut-brain axis studies on the infant population are heavily lacking. To date, only one study by Carlson et al. (2018) that directly evaluated the microbiota-gut-brain interplay focusing on the early brain and cognitive development in infants. They suggest that microbial diversity may affect cognitive function in early life and showed that cognitive development at two years of age could be predicted from the microbial diversity at one year of age. Additionally, they reported structural brain differences by which alpha diversity showed an association with several regional gray matter volumes. However, they showed no association at neither one nor two years of age between alpha diversity and the caudate nuclei.

Contrary to our hypothesis, the presented results did not evince the presence of a relationship between infant gut microbiota diversity and the caudate nuclei and putamina. Diversity is a standard measure used to characterize the microbiota, and the alpha diversity assesses the mean bacteria species diversity within a sample and is expressed through indices (Hagerty et al., 2020, Kelsey et al., 2019). This study used the Shannon index of alpha diversity in our statistical analysis, and internal and external influences on the gut microbiota and/or the infant's brain were taken into account as confounding variables for all statistical testing. The gut microbiota alpha diversity was tested for association with left and right hemispheres caudate nucleus and putamen using a Spearman's correlation, resulting in an insignificant and low correlation coefficient. These results are consistent with the findings of Carlson et al. (2018). Besides, exploring sex differences in the association between alpha diversity and striatum volumes was insignificant, despite visually demonstrating a stronger association in girls. Furthermore, the calculation of the beta dispersion (compositional heterogeneity in microbiota by measuring variance of the distance to the group centre) and beta diversity (compositional similarity of microbiota) for the population were performed but did not demonstrate any significant effect size when tested caudate nuclei and putamina. Also, the differential abundance showed an absence of significant microbial signature within our sample regarding the left and right caudate nucleus and putamen.

The paucity of studies conducted on infants limits our ability to validate and interpret our results against previous findings. A considerable number of past gut-brain axis studies focused their attention on crucial cerebral components involved in various early cognitive developmental processes (e.g., attention, emotion, or temperament) or neurodevelopmental disorders (e.g., ADHD or autism). For instance, the amygdala, a crucial subcortical structure for emotional and cognitive processing (e.g., fear), has been reported to be associated (i.e., volume and functional connectivity) with infant gut microbiota diversity (Carlson et al., 2018; Gao et al., 2019). The amygdala volume and functional connectivity appeared to be associated with alpha diversity (Carlson et al., 2018; Gao et al., 2019) negatively. Moreover, findings by Christian et al. (2015) attest of a correlation between the gut microbiota beta diversity and fear reactivity, a response involving the amygdala. Together, these results show the gut microbiota may be associated with specific subcortical structures. Hence, we hypothesized that the amygdala results could be extrapolated to other subcortical structures, such as the striatum. However, our results are contrary to this hypothesis, but further research is needed.

Arguably, the absence of a significant association between microbiota diversity and striatum volumes might be explained by the temporal variation in microbial diversity and the PNI pathways development (Jena et al., 2020; Stewart et al., 2018). The population-level analysis of gut microbiome variation by Falony et al. (2016) yields indication of incomplete total gut diversity in infancy. This may be due to the fact that microbiota diversifies through the first year of life, especially at dramatic periods of change in diet such as weaning and the introduction of solid foods (Stewart et al., 2018). Nevertheless, the role of early microbiota diversity on later health remains to be elucidated. Presumably, a more diverse microbial community usually concords with better health in adults (Menni et al., 2017; Hooks et al., 2018; Shade et al., 2017); thus, the same could be inferred for infants.

Additionally, the gut-brain communication pathways are still in their infancy during early life. For instance, the vagus nerve, critical in gut-brain neural communication, is still not fully myelinated in early infancy. Additionally, the immune defence for the body during infancy is provided through the mother's milk high in level of immune mediators. Indeed, the relatively immature immune system in the first months, or years of life, develops via stimulation by the gut microbiota community. Hence, the immune and neural pathways critical for gut-brain communication are not fully developed and functional in early life. This lack of development might reduce the influence of the gut microbiota on brain structural and functional development. Or rather, it could be hypothesized that the influences of the gut microbiota, or other early life environment exposures, are only visible later in infancy of childhood.

The microbiota, in addition to temporal variation, also has spatial and potential sexual variability. The intestinal microbiota composition varies along the gastrointestinal tract's length, with the highest diversity in the colon (Tropini et al., 2017; Jena et al., 2020; Sekirov et al., 2010). Fecal sampling provides us with information regarding the microbial community present in the colon's lumen, which is more abundant than the mucosal microbiota (Ringel et al., 2015; Tropini et al., 2017). Further gut-brain research should implement a combinatory sampling from the luminal and microbiota to show the entire spectrum of microorganisms living in our gut. However, sampling mucosal microbiota requires an intestinal wall biopsy, an invasive procedure that is impractical for infant population studies. Sex variability is another factor that has been suggested to affect the gut-brain axis. For instance, gender-specificity has been shown in neurochemical and endocrine effects of gut microbiota, anxiety behaviour or social development (Cryan & O'Mahony, 2011; Neufeld et al., 2011; Desbonnet

et al., 2014). Besides, a study by Acosta et al. (2020) observed sex-specificity for caudate volumes association with the genetical risk for major depressive disorder. The study demonstrated a stronger positive association in boys than girls. However, we visually observed a small sex-dependent difference in the microbiota-striatum volumes associations, yet not significant. This lack of significance of sex-specificity in the association between gut-microbiota and striatum volumes appears to be at odds with previous literature suggesting sex variability. However, it could be a reflection of the low statistical power of the current study leading to the impossibility of detecting such a small to moderate effect. In all, further gut-brain studies focusing on gender-specificity are necessary to determine the translatability of preclinical results in humans.

5.2 Limitations

Particular limitations in this exploratory study needs to be acknowledged. The following are developed: statistical power, confounding factors, temporality and single time point assessment, and gut microbiota metrics.

5.2.1 Statistical power

This study consisted of a sample of 56 infants. Several factors directly influenced the infant's gut microbiota and brain volume variability and were considered as covariate variables. However, the statistical power may have been inadequate in detecting a small effect even if one was present, as it would have been limited. A post hoc investigation of the statistical power of our study demonstrated that in order to detect the potent effect of gut microbiota of the striatum with a power of 80%, we would need a population size of at least twice the current size (~120) for a medium effect or even twelve times higher (~700) for a small effect. Indeed, as the population size increases, the effect size that can be detected becomes smaller. Moreover, a statistical power of 80% was advanced as the desired power in research by Cohen (1992), but due to the paucity of literature on the subject, and with this specific design in this age group, the population size needed for sufficient statistical power could not be determined before the study. Additionally, the population size was limited for practical reasons, as only 56 infants provided a fecal sample from the ~200 infants who underwent the MRI scanning. Therefore, this study only had enough statistical power to detect large effects, so forthcoming studies must favor larger sample size, when possible, to optimize statistical power in order to observe a small to moderate effect size.

5.2.2 Confounding factors

The microbiota composition is significantly influenced by breastfeeding and delivery mode (Stewart et al., 2018; Borre et al., 2014), as a TEDDY study showed associations between breastfeeding and high levels of Bifidobacterium species as well as vaginal birth and high levels of Bacteroidetes (Stewart et al., 2018). Indeed, vaginal and cesarian deliveries expose the infant to the different microbial milieu, correlating with gut microbiota diversity (Stewart et al., 2018) and later developmental outcomes (Borre et al., 2014). Infant delivered by the cesarian section shows a higher risk to suffer from allergies, gastrointestinal dysfunction or diabetes later in life (Jakobsson et al., 2014; Domingez-Bello et al., 2010). In addition to delivery mode, genetic and environmental factors also help define the microbiome. The microbiome has been suggested to cluster within families but with significant contribution from environmental factors over genetics (Lozupone et al., 2012; Maynard et al., 2012). Similar to birth mode, infants born prematurely lack two of the major bacterial genera seen in infants born at term, although they were acquired through breastfeeding (Barrett et al., 2013). Also, studies showed stabilization in the microbiota composition through breastfeeding, contrary to formula feeding, which leads to higher diversity (Fan et al., 2014, Roger et al., 2010). Besides, breastfeeding cessation is a determinant for adult-like microbiota maturation in late infancy (Bäckhed et al., 2015). Therefore, in this study, covariate variables included infant sex, birth mode and feeding mode at fecal sample. Diversity, precisely beta diversity, exhibited a correlation with birth mode and child sex, but not with feeding mode. However, the majority of the infant population were vaginally delivered (80%) and breastfeed (77%), so the sample was relatively homogenous and could not represent all the possible delivery and feeding status (e.g., cesarian or formula feeding) evenly. Other factors, including siblings and antibiotic treatments, could have been considered, as they have been suggested to associate with microbiome profiles and maturation (Stewart et al., 2018; Bokulich et al., 2016). Yet, these studies are focusing on children, not infants, so it is conceivable that these factors (i.e., sibling and antibiotics) may play a more important role in later microbiota composition as the number of antibiotic treatments in the first few months of life is generally lower than during toddlerhood or childhood. Additionally, siblings and antibiotic treatments are associated with the gut microbiota and might not influence striatum volumes.

In addition to confounding factors influencing gut microbiota, brain volumes (e.g., striatum or amygdala) might be influenced by internal and external factors, including maternal health, medication exposure and genetics. Studies showed the influence of genetic variation

and environmental and epigenetic factors on amygdala volumes (Ong et al., 2019; Satizabal et al., 2019). Hence, infant medications (e.g., antibiotics) and maternal medication (e.g., anti-depressants) could have been taken into account, as it could be hypothesized that they may influence striatal volumes similarly to amygdala volumes (Aatsinki et al., 2020). However, the influence of genetic variation on early microbiota association with brain structural and functional development should be explored with further research.

5.2.3 Single time point assessment and temporality

The age group of the population is more challenging when measuring gut microbiota properties, such as diversity, as infants undergo swift gut microbiota colonization and maturation all along the first years of life until the age of 3 years (Jena et al., 2020; Stewart et al., 2018; Borre et al., 2014). Indeed, the microbiota flourishes into an intricate, adult-like composition characterized by higher alpha diversity and lower beta diversity (Bäckhed et al., 2015; Yatsunenko et al., 2012). Similarly, brain development is vital during the first years of life, with significant cognitive, emotional and structural changes (Borre et al., 2014; Carlson et al., 2018; Gilmore et al., 2012; Knickmeyer et al., 2008; Provost, Hanganu, and Monchi, 2015). Hence, together with the considerable changes in fecal microbial composition and diversity at weaning and the introduction of solid food (Borre et al., 2014), the presumed connection between gut microbiota and the brain may only develop later during infancy, i.e., post weaning. Or rather, it could be also developed that the gut-brain connections may represent differently in later timepoints of early life (e.g., toddlerhood), than during infancy. Therefore, to fully understand the gut-brain associations over the course of early life, from birth to the end of childhood, multi-time point assessments study design should be considered in future research.

Several limitations in our study arise from the rapid evolution of the intestinal microbiota and brain. First, both the gut microbiota and striatum volumes were assessed by a single time point sample at respectively 2.5 months and one month approximatively. Secondly, the temporal distance of approximately five weeks between the MRI measurements and fecal sample might be a great source of variability, especially regarding the gut microbiota. Indeed, as mentioned previously, the microbiota has a rapid evolution, and the important temporal difference between the two samples may be a source of inaccuracy in the gut microbiota characteristics. Furthermore, fecal sampling was done after the infant's MRI imaging, so the infant gut microbiota at 2.5 months of age might be reasonably different –

more developed – than at one month of age when undergoing imaging. This temporal difference highlights the dual impact of time on the information extracted from the gut microbiota composition and the brain imaging and the resulting observation of associations. Hence, it should be noted that our results only describe a snapshot of the possible associations during infancy, so further studies are needed. Importantly, studies with longitudinal designs will provide a more substantial report concerning the parallel gut microbiota and brain development and interactions in early life.

5.2.4 Gut microbiota diversity indexes

This thesis implemented a narrowed view of the gut microbiota, as diversity parameters might be over-simplistic to characterize fecal microbiota. Indeed, a study by Shade (2017) points out that microbial diversity calculations possess biases and thus should be used as rough approximations as they are not absolute values. In this study, we solely used the Shannon index as a representation of alpha diversity. Previous studies often use four different indices to assess alpha diversity, including observed species, Faith's Phylogenic Diversity, and Chao1 (Gao et al., 2019; Carlson et al., 2018). Together, they provide information about taxonomic and phylogenic composition, richness, and evenness in the sample (Navas-Molina, 2013; Gao et al., 2019). In this study, the Shannon index of alpha diversity provides information on the fecal microbiota community's richness and evenness. Therefore, future studies should implement a combination of alpha diversity indices to get a more global picture of the associations between microbiota diversity and brain structures. Moreover, the diversity and abundance measures performed in this study fail to appreciate the functional properties of gut microbiota, which may be crucial in gut-brain communication. Consequently, a complementary assessment of gut microbiota structure and functionality via a two-stage experiment combining 16S rRNA sequencing and shotgun sequencing (description of the functional potential of microbiota) would provide information about both the gut microbiota structure and its metabolites' influences on brain neurodevelopment (Morgan & Huttenhower, 2014; Tickle et al., 2013).

5.3 Future research

Future studies are critical in enriching the limited literature concerning infant gut-brain axis. Exploration of whether and how the gut microbiota composition, diversity and functionality correlate with the brain structural, cognitive and behavioural development in

infant would elevate the current understanding of the origins of the gut-brain axis. Besides, reporting null or negative results in published literature would permit development of interest amongst researchers as well as improvement of study designs to confirm or infirm the absence of correlations between gut microbiota and brain development in infancy. Furthermore, future study designs should draw methodological improvements from the conclusions and limitations of our novel study. As such, a longitudinal design of a large infants' cohort with consideration of broader confounding factors and diversity indexes and both structural and functional aspects of neurodevelopment, may provide sufficient statistical power and temporality to observe a significant correlation (small or moderate) between the gut microbiota and the brain. In all, future insight in gut-brain relationship during infancy will add tremendously to the literature.

6 Conclusion

This thesis explored the potential association between striatum volumes and gut microbiota diversity. The present findings only present a snapshot of the gut-brain relation in infancy. Despite the lack of clear associations, this thesis added to the currently limited literature investigating the origins of the microbiota-gut-brain axis communication. Importantly, further studies exploring neonatal gut microbiota and its associations with brain structural and functional development would provide essential information on the parallel gut and brain development interplay. Overall integrity in brain structure and function is vital for our well-being throughout life, so it is important to understand how external factors, such as gut microbiota, influences early life neurodevelopment. Further studies are important to examine how the gut microbiota composition, diversity and functionality associate with brain structures or processes. Additionally, longitudinal research is needed to provide insight into how gut microbiota associates with the brain during the critical period of neurodevelopment throughout life.

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