NUTRITION AND THE GI MICROBIOTA IN CHILDREN WITH AUTISM SPECTRUM DISORDER AND IMPACT ON SYMPTOM SEVERITY

BY

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DISSERTATION

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ABSTRACT

The gastrointestinal (GI) microbiota is increasingly recognized for its ability to influence brain function and behavior. In children with Autism Spectrum Disorder (ASD), a microbial dysbiosis has been described and some bacterial taxa were found to predict certain symptoms of ASD. Additionally, picky eating behavior and food aversions are common in children with ASD, resulting in limited diet variety and decreased nutrient intake (e.g., fiber). Diet is one of the major determinants of the GI microbiota; however, previous studies have not systematically investigated the role of diet in shaping the GI microbiota in children with ASD. Likewise, overall microbial stability is recognized as more beneficial due to its ability to protect against pathogen invasion and maintain overall function. In children with ASD little is known about the stability of the microbiota. Therefore, *the goal this dissertation research was to assess the impact of diet on the GI microbiota in children with ASD and microbial stability over a 6-month period* with the following aims :1) to investigate differences in microbiota composition and volatile fatty acid (VFA) concentration between children with ASD and unaffected controls and investigate the relationship to ASD symptoms; 2) to determine the effect of long-term dietary patterns and short-term nutrient intake on the fecal microbial composition and VFA concentration in children with ASD and uncover relationships between diet, fecal microbiota, VFAs and ASD symptoms; and 3) to analyze the microbiota composition and VFA concentrations in children with ASD and unaffected controls over a 6-month period and identify dietary factors that correlate with a more stable microbial profile.

Children with ASD (ASD; n=26) and age- and sex-matched unaffected controls (CONT; n=32) were recruited in the Midwest area. Fecal samples, a 3-day food diary, a food frequency questionnaire, and an online questionnaire collecting information on demographics, GI health,

nutrition supplement use were collected at baseline, 6-weeks post-baseline and 6-months post baseline. ASD symptoms were assessed using the Pervasive Developmental Disorder Behavior Inventory – Screening Version (PDDBI-SV). Bacterial DNA was analyzed using 16S rRNA sequencing and quantitative Polymerase Chain Reaction. VFA concentrations were analyzed by gas chromatography. Dietary patterns were derived from the Youth and Adolescence Food Frequency Questionnaire (YAQ) using Principal Component Analysis and exploratory Factor Analysis. Nutrient intake was assessed by the Nutrition Data System for Research. All data were analyzed using SAS 9.4.

Differences in microbiota composition between ASD and CONT were observed. Overall, β-diversity assessed by permutational multivariate analysis (PERMANOVA) differed ($p=0.02$) based on unweighted but not weighted d UniFrac. α-diversity measured as observed Operational Taxonomic Units (OTUs) tended to be higher ($p=0.08$) in ASD. Microbial abundances on the phyla, family, order and genera level were observed. Namely, ASD had higher levels of Firmicutes, Clostridiales, Clostridiaceae, Peptostreptococcaceae, Coriobacteriaceae, *Clostridium*, *SMB53*, *Blautia*, and *Roseburia*, but lower levels of Bacteroidetes, Streptophyta, Rikenellaceae, *Butyricimonas*, *Butyrivibrio*, *Faecalibacterium*, *Dialister*, and *Bilophila* compared to CONT. Furthermore, higher concentrations of acetate, propionate and butyrate were detected in ASD. Lastly, Peptostreptococcaceae and *Faecalibacterium* predicted social deficit (SOCDEF) scores in children with ASD as measured by the PDDBI-SV.

Investigating dietary intake revealed that children with ASD consumed lower amounts of insoluble fiber, pectin, vitamin C and dairy, but consumed more snacks and sweets than unaffected children. To analyze the impact of nutrition on the GI microbiota, four analyses were utilized. First, correlation analyses revealed that nutrient and food group intake were associated

with the abundance of bacterial taxa. Second, children with ASD characterized as picky eaters or having a repetitive eating pattern harbored a unique microbial composition. Third, two dietary patterns (DP) were empirically derived for children with ASD using the YAQ. DP-1, characterized by intakes of vegetables, starchy vegetables, legumes, nuts and seeds, fruit, grains, juice and dairy, was associated with lower abundance of Enterobacteriaceae, *Lactococcus*, *Roseburia*, *Leuconostoc*, and *Ruminococcus*. DP-2, characterized by intakes of fried foods, Kid's meals, condiments, protein foods, snacks and starchy foods, was associated with higher abundance of Barnesiellaceae, *Alistipes*, and lower abundance of *Streptophyta* as well as higher concentrations of propionate, butyrate, isobutyrate, valerate, and isovalerate. Diet-induced microbial comppsition was related to some GI symptoms, but was not related to SOCDEF scores. Lastly, moderation analysis did not reveal a significant interaction between microbial taxa and dietary components in prediction SOCDEF scores.

To investigate the temporal microbial stability, two additional samples collected over a 6-month period were analyzed for microbiota composition and VFA concentration. We found that overall the microbiota composition and metabolites concentration varied in children with ASD and variability in community membership negatively correlated with median SOCDEF scores. Furthermore, different bacteria taxa contributed to a stable microbiota profile in each group. Clostridiales, Ruminococcaceae, *Lactococcus*, *Turicibacter*, *Dorea*, and *Phascolarctobacterium* contributed to a more stable microbiota community in children with ASD whereas Barnesiellaceae, *Adlercreutia*, *Faecalibacterium*, *Sutterella* and *Bilophila* contributed more in to a stable microbiota in CONT children. Lastly, GI microbiota variability was related to habitual dietary patterns.

Overall, the results presented herein contribute to the growing literature on a microbial dysbiosis and the impact of specific microbial taxa on symptoms in children with ASD. Importantly, this research offers new insight into the effect of diet on the microbiota composition, microbial metabolism and the temporal variability in children with ASD. Future studies are warranted to analyze whether dietary intake could potentially be a modifiable moderator of the microbiota-symptoms connections in ASD.

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CHAPTER 1
Introduction

Autism spectrum disorder (ASD) is typified by deficits in social communications skills and the presence of repetitive or restrictive behaviors (APA, 2013). In 2014, 1-in-59 children in the United States were diagnosed with ASD and there is a continuing upward trend in the number of children being diagnosed with ASD (CDC, 2014). Whether this increase is due to enhanced clinical testing and diagnostic methods, broader diagnostic criteria or increased environmental risk factors is unknown. Identifying causes underlying the development of ASD is challenging due to difficulties in stratifying the ASD population due to the phenotypic heterogeneity of the disease and occurring comorbidities (e.g. anxiety, hyperactivity); however, genetic as well as environmental factors are suggested to play a role (Hallmayer et al., 2011). Because genetic factors might account for only 10-20% of the observed ASD cases and concordance rate of monozygotic twins is less than 100% (77% for male and 50% for female pairs), importance of environmental elements, including the gastrointestinal (GI) microbiota and nutrition, in contributing to ASD symptom development has be suggested (Hallmayer et al., 2011).

Increasing evidence is presented for the ability of the GI microbiota to influence cognitive function and behavior via the "microbiome-gut-brain axis" (Cryan & Dinan, 2012; Bienenstock et al., 2015). Differences in fecal microbiota composition at the phyla and genus level between children with ASD and unaffected controls have been documented (Finegold et al., 2010; De Angelis et al., 2013; Kang et al., 2013). Furthermore, specific bacterial taxa have been shown to be associated with the severity of some symptoms of ASD. For example, a lower ratio of Bacteroidetes-to-Firmicutes and a higher abundance of *Clostridium* and *Desulfovibrio* were

positively associated with severity of ASD symptoms (Tomova et al., 2015). Besides differences in the fecal microbiota, deviations in microbial products between children with ASD and unaffected controls have also been observed, suggesting that some microbial products could be a way of communication between the GI microbiota and symptoms of ASD (Wang et al., 2012). Lastly, intervention studies showing that some probiotics can alleviate some symptoms of ASD provide additional evidence for a microbiota-brain connection in ASD (West et al., 2013).

Emerging research also supports a potential role of nutrition in cognitive function. For example, in healthy children an interrelationship between dietary intake, neurodevelopment, and cognitive function has been reported (Khan et al., 2015a), suggesting an important role of diet quality on brain function. Achieving adequate nutritional intake in children with ASD is a challenge due to GI symptoms or problematic eating behaviors (Liu X et al., 2016). Food selectivity and aversions with lower intakes of fruits and vegetables are commonly reported (Cermak et al., 2010) which can lead to malnutrition and nutrient deficiencies (Ledford & Gast, 2006).

The GI microbiota is influences by a variety of environmental factors, including diet. It has been estimated that more than 50% of microbial changes can be attributed to diet (Zhang et al., 2010). Thereby, long-term dietary patterns as well as short-term nutrient intake can determine the composition and function of the GI microbiota (Berding et al., 2018, David et al., 2014). Diet-induced changes in microbiota composition can lead to increased risk of developing certain diseases (e.g., inflammatory bowel diseases), whereas a healthier long-term dietary pattern may be more beneficial in promoting a microbial profile that could potentially protect against diseases (Albenberg & Wu, 2014). The GI microbiota undergoes changes during early life and becomes relatively stable at 3 years of age. However, it is now increasingly recognized

that the microbiota composition can undergo temporal variations over weeks or even the course of the day (Kazcmarek et al., 2017; Caporaso et al., 2011; Flores et al., 2014). Thereby, a stable microbiota with high levels of commensal bacteria could be indicative of health due to the ability to protect against pathogen invasion and maintain functions (Galloway- Peña et al., 2017; Ley et al., 2008). Greater microbial instability was previously found in patients with intestinal diseases such as Inflammatory Bowel Syndrome of Chron's disease (Mättö 2005; Maukonen et al., 2006; Scanlan et al., 2006).

Although the impact of diet on the microbiota composition is well described and the nutritional intake of children with ASD is often limited, most of the research published to date analyzing the GI microbiota in children with ASD lacks in providing substantial information on dietary intake in the study population. Additionally, previous research lacks in informing about the temporal dynamics of the microbiota composition in the ASD population and the potential impact of dietary factors on the variability. Thus, this dissertation research presents a new approach at investigating the microbiota-ASD connection by systematically analyzing the microbiota composition in the context of dietary intake, by examining the potential moderating effect of diet on the microbiota-ASD interaction and by determining the impact of diet on long-term variability in children with ASD.

1.1 Objective

The ***long-term goal*** of this research is to develop evidence-based recommendations to alleviate symptoms of ASD symptoms through manipulation of the gut microbiota. Thus, the ***overall objective*** of this dissertation research was to 1) systematically describe the influence of diet on the interactions between the GI microbiota and symptoms of ASD and to 2) define the

relationship between diet and longitudinal microbial dynamics in children with ASD and the association with ASD symptoms. The ***central hypothesis*** is that diet-induced microbial profiles will be associated with ASD symptom severity and long-term dietary patterns will determine microbial stability in children with ASD. The ***rationale*** is to gain a better understanding of the interplay between diet, microbiota and severity of ASD symptoms in order to determine whether manipulation of the GI microbiota could be used as an effective intervention option for management of symptoms of ASD.

1.2 Specific Aims

Specific Aim 1: Analyze the fecal microbial composition and VFAs concentration in children with ASD and unaffected controls and determine association with symptom severity.

The ***overall objective*** of this aim is to characterize the microbial profile and bacterial metabolic activity of children with ASD. Our ***working hypothesis*** is that microbial composition between children with ASD and unaffected controls will differ in overall diversity and composition (e.g. Bacteroidetes-to-Firmicutes ratio) and specific bacterial taxa will predict severity of symptoms of ASD. We ***further hypothesize*** that VFA (volatile fatty acid) concentrations will be greater in children with ASD and will be associated with higher propionate concentrations relative to acetate and butyrate.

Specific Aim 2: Uncover relationships between diet, fecal microbiota, VFAs and ASD symptom severity.

The ***overall objective*** of Aim 2 is to characterize dietary patterns or specific nutrients that drive a microbial profile associated with symptom severity in children with ASD. The ***working hypotheses*** are that 1) dietary patterns (i.e. fruit and vegetables vs. processed foods) and nutrient intake (e.g. dietary fiber) will determine the GI microbiota compostion in children with ASD and that 2) a microbial profile and VFA concentrations induced by specific dietary patterns and nutrient intake will predict symptoms of ASD.

Specific Aim 3: Determine long-term variability of microbiota composition in children with ASD and association with dietary patterns.

The ***overall objective*** is to investigate potential changes in the GI microbiota composition in children with ASD. The ***working hypotheses*** are that 1) the microbiota composition of children with ASD will be more variable compared to unaffected controls and will be associated with more severe ASD symptoms and that 2) dietary pattern characterized by higher intakes of fruit and vegetables will be associated with a more stable microbial profile compared to a dietary pattern high in processed foods.

This dissertation research provides new evidence for a microbial dysbiosis in children with ASD and fills the gap in the existing literature on the impact of diet on the microbiota composition in children with ASD. Understanding the impact of diet on the GI microbiota in children with ASD can provide a significant step towards developing targeted dietary or prebiotic intervention strategies to alleviate some symptoms of ASD. Within this dissertation, Chapter 2 provides a literature review in the microbiota, nutrition and microbiota-gut-brain axis in ASD and the potential moderation of nutrition on the microbiota-brain connection. Chapter 3

includes how long-term dietary patterns influence the microbiota composition and longitudinal variability in healthy children. Chapter 4 describes the differences in microbiota composition and VFA concentrations between children with ASD and unaffected controls and the impact of specific bacterial taxa on ASD symptoms (Aim 1) Chapter 5 systematically examines the relationship of diet and the microbiota composition in children with ASD and the association with ASD symptom severity (Aim 2). Chapter 6 analyzed the longitudinal variability of the microbiota in children with ASD and unaffected control and determines the impact of dietary patterns (Aim 3). Finally, chapter 7 offers overall conclusions and future directions from this research.

CHAPTER 2

Literature Review[1]

2.1 Introduction

Autism spectrum disorder (ASD) is typified by deficits in social communications skills and the presence of repetitive or restrictive behaviors (APA, 2013). The term "spectrum" refers to the wide variety in symptoms and intensity of symptoms and includes Autistic Disorder, Pervasive Developmental Disorder Not Otherwise Specified and Asperger Syndrome (APA, 2013). In 2014, 1-in-59 children in the United States were diagnosed with ASD and there is a continuing upward trend in the number of children being diagnosed with ASD (CDC, 2014) (**Figure 1.1**). Between 2000 and 2010, ASD diagnoses have double from 6.7 to 14.7 per 1000 children aged 8 and under (Baio, 2012). Whether this increase is due to enhanced clinical test and diagnostic methods, broader diagnostic criteria or increased environmental risk factors is unknown. Identifying causes for the development of ASD is challenging due to difficulties in stratifying the ASD population because of the phenotypic heterogeneity of the disease and occurring comorbidities (e.g. anxiety, hyperactivity); however, genetic as well as environmental factors are suggested to play a role (Hallmayer et al., 2011). Because genetic factors might account for only 10-20% of the observed ASD cases and concordance rate of monozygotic twins is less than 100% (77% for male and 50% for female pairs), importance of environmental elements, including the GI microbiota and nutrition, in contributing to ASD symptom development has be suggested (Hallmayer et al., 2011). Additionally, only half of the recent increase in incidence of ASD could be explained by known factors (e.g. genetics, awareness,

[1] A portion of this literature review was previously published in Nutrition Reviews, entitled "Microbiome and nutrition in autism spectrum disorder: current knowledge and research needs".

improved clinical testing). Approximately 46% are left unexplained which could be accounted for by unknown environmental factors (Nardone et al., 2014).

Food selectivity, food neophobia, and "picky eating" are prevalent among children with ASD and can contribute to the development of nutrient deficiencies (Marí-Bauset et al., 2013). Indeed, vitamin, mineral and fatty acid deficiencies have been observed in children with ASD, including deficiencies in vitamin D and calcium (Ranjan & Nasser, 2015). Because parents are concerned about the nutritional status of their children and side effects of traditional medication, Complementary and Alternative Medicine are commonly used by parents of children with ASD (Levy & Hyman 2015).

The microbiome-gut-brain axis has been described as a way of communication between the gut microbiota and the brain (Bienenstock et al., 2015). Functional and inflammatory gastrointestinal (GI) diseases show a high co-morbidity (94%) with psychiatric diseases, such as depression or anxiety (Whitehead et al., 2002). On the other hand, compositional differences in the GI microbiota of patients with neurodevelopmental disorders, including ASD, have been reported (Finegold et al., 2010; Parracho et al., 2005) Alleviation of ASD symptoms after probiotic treatment highlights the potential of the GI microbiota to influence the symptoms associated with ASD (Critchfield et al., 2011). Likewise, studies have begun to investigate the potential of dietary interventions in managing diseases with an underlying gut-brain connection.

Although dietary problems are well documented in children with ASD, studies investigating the GI microbiota composition thus far have not paid much attention to dietary impact on the GI microbiota in children with ASD. To further understand the potential of manipulating the GI microbiota and alleviating some symptoms of ASD, it is important to delineate the impact of dietary patterns and nutrient intake on the GI microbiota composition.

The goal of this review is to summarize the current evidence base regarding the GI environment and the nutritional status of children with ASD. Potential underlying mechanisms of the microbiome-gut-brain-axis in ASD and interplay between nutrition, microbiota and ASD symptoms will also be reviewed.

2.2 Diagnostic Criteria and Treatment Recommendations

Diagnostic Criteria

A number of tools are available for ASD screening and diagnosis. These instruments differ in specificity of the appropriate age range, length of administration and method of administration. For example, different screening tools are available for parents, teachers/caregivers and clinicians. Examples for screening scales include Autism Behavior Checklist (ABC) or Autism Spectrum Quotient (ASQ). Most commonly used diagnostic scales include Autism Diagnostic Interview – Revised (ADI-R) and Autism Diagnostic Observation Schedule (ADOS). Although the tools available for ASD diagnosis show a high level of specificity and sensitivity, the phenotypic heterogenic nature of ASD makes a clinical multidisciplinary team assessment, i.e. including both qualitative and quantitative data from multiple sources, important for a more comprehensive and accurate diagnosis (Le Couteur et al., 2008). Correctness of diagnosis with multidisciplinary team approach for ASD is approximated at 80.8% (Falkmer et al., 2013).

Diagnostic tools used in the clinical setting are guided by the Diagnostic and Statistical Manual of Mental Disorders (DSM) which is published by the American Psychiatric Association (APA). According to the DSM 5th edition, to be diagnosed with ASD, the child has to show deficits in following areas (APA, 2013):

"a) deficits in social communication and social interaction (social-emotional reciprocity, nonverbal communicative behaviors used for social interaction, developing, maintaining and understanding relationships)

b) restricted, repetitive patterns of behavior, interests or activities manifested by at least 2 of the following: stereotyped or repetitive motor movements, use of objects or speech; insistence on sameness, inflexible adherence to routines or ritualized patterns of verbal or nonverbal behavior; highly restricted, fixated interests that are abnormal in intensity or focus; hyper- or hypoactivity to sensory input or unusual interest in sensory aspects of the environment,

c) symptoms must be present in early development period;

d) symptoms cause clinically significant impairment in social, occupational or other important areas of current functioning,

e) disturbances are not better explained by intellectual disability and global developmental delay"

In order to provide a more reliable diagnosis of ASD and assist in targeted intervention strategies, an increasing number of investigators are exploring the use of biomarkers for ASD diagnosis. These markers include biochemical (e.g. serotonin levels), immunological (e.g. inflammatory cytokines), hormonal (e.g. oxytocin) or functional markers (e.g. brain connectivity) (Fakhoury, 2015). However, translating the discovery of biomarkers into the clinical application is challenging and none of the biomarkers proposed for ASD has yet been validated for clinical use.

Treatment

Early diagnosis and intervention has been proven to be important in providing better short- and long-term outcomes for the family and the affected child. For example, secondary symptoms, such as disruptive behavior, and other comorbidities (e.g. anxiety) were shown to be decreased in children receiving early intervention (Koegel et al., 2014). The health care cost for a child with ASD is 6-times higher compared to that of an unaffected child (Shimabukuro et al., 2008). Once a child has been diagnosed with ASD, treatment and interventions usually last for the entire duration of their lives. Over the life of an affected child, the health care cost, e.g. costs for educational services, parental productivity loss and medical costs, can go up to $1.4 million per family (Buescher et al., 2014). Due to the heterogeneity of symptoms, treatment among individuals varies significantly and no single medicine or therapy is effective in treating all symptoms. A multimodal approach is most likely to promote optimal treatment.

The pharmacological treatments available for ASD do not specifically target the "core" symptoms of ASD (impaired social interaction, communication and imaginative play and restrictive, repetitive activities and interests), but rather are targeting problematic "associated" behaviors (hyperactivity/inattention, aggression, motor stereotypes) (West et al., 2009). Examples for medications used in the ASD population include antipsychotic agents (e.g. risperidone, methylphenidate (MPH) and atomoxetine) antidepressants (e.g. selective serotonin reuptake inhibitors (SSRIs)) and anticonvulsants (Hirota et al., 2014). Some pharmacological treatment is used in conjunction with behavioral and educational therapy to increase the effectiveness.

Risperidone and aripiprazole are the only drugs approved by the FDA and are usually the first-choice agents to treat ASD symptoms (Erickson et al., 2010, McKinney & Renk, 2011). These

atypical neuroleptics mostly target dopaminergic or serotoninergic systems and are used for irritable, aggressive or self-injurious behavior, in children and adolescents with autism. Recent reviews of randomized controlled trials using risperidone found that most studies reported an improvement in associated behaviors of autism (Canitano & Scandurra, 2011; West et al., 2009). Studies investigating the effect of MPH and atomoxetine, mostly treating hyperactivity and attention disorder, reported differing levels of improvements (Canitano & Scandurra, 2011). Moderate responses have been observed with SSRIs, which commonly treat deficits in socialization and communication as well as aggression and repetitive behaviors; however, its use is not generally recommended (Canitano & Scandurra, 2011; West et al., 2009). Evidence presented for the use of anticonvulsants is limited and thus far does not appear to provide a large size effect on behavioral symptoms (Hirota et al., 2014).

Although there is limited knowledge on the effect of pharmacological treatment, almost half of patients are taking some form of medication with other studies reporting even higher percentages (Aman et al., 2003; Coury et al., 2012). When stratified by age, one study found that the percentage of children taking medication with age and reaches about 75% at age 12 years (Coury et al., 2012).

In general, pharmacological treatment for the pediatric ASD population is not well studied. Studies published to date have shown more success in treating associated behaviors compared to core symptoms. Developing targeted treatment options is made more difficult by the missing knowledge of the exact neuropathology of ASD. Likewise, the effects of medicine on the developing brain are unknown and potential neurobiological abnormalities need to be investigated. Therefore, it is generally recommended to only use medication when behavioral

interventions have failed and when physical risks due to associated behaviors outweigh potential side effects from medication (McKinney& Renk, 2011).

Behavioral and Educational Intervention: Early intensive multidisciplinary behavioral intervention is recommended before considering pharmacotherapy for improvement of behavior. The literature has shown that most families adopt multiple treatment approaches in order to alleviate symptoms (Bowker et al., 2011). Parents mostly learn about treatment options from the internet, professionals or occupational therapists (Green, 2007).

Behavioral interventions are usually started right after diagnosis and are the most common treatment approaches for ASD. A large amount of interventions available for ASD are based on methods derived from the applied behavior analysis (ABA) which is the most validated approach for education and treatment of ASD (Manning-Courtney et al., 2003). Examples of behavioral interventions include developmental interventions to improve cognitive performance, language skills and adaptive behavior, social skill intervention or play-/interaction-based intervention.

Educational interventions traditionally focus on areas of academic progression and also address core areas of social, cognitive and behavioral deficits via the classroom or specialized institutions. One of the most widely known approaches to educational intervention in children with Autism is the TEACCH (Treatment and Education of Autistic and related Communication Handicapped Children) Method (Gerlach, 2003). The evidence presented to date has shown improvement in functioning of a subgroup of ASD children using of educational and behavioral intervention. Education is lifelong process, should begin as early as possible. Goals for educational intervention include enhancing social skills, improving language skills, fine and gross motor skills, cognitive skills (Lord & McGee, 2001).

Other forms of nonpharmacological therapy included speech and language therapy to develop language function and a functional communication system as well as occupational therapy to regulate sensory systems and promote fine motor skills (Manning-Courtney et al., 2003)

Complementary and Alternative Medicine: National Center for Complementary and Integrative Health (NCCIH) defines two domains of CAM: natural products and mind and body practices (NCCIH, 2014). Natural products include herbs, vitamins and mineral or probiotics. Mind and body practices include yoga, meditation or massage therapy. Interestingly, parents learned about CAM through family or community, nonmedical professionals or internet and rarely asked physician for information (Wong & Smith, 2006). Additionally, parents with higher degree and higher socioeconomic status are more likely to use CAM (Wong & Smith, 2006; Akins et al., 2014).

Although only a very little amount of high quality research is published to support use of CAM in management of ASD, reported percentages of parents of children with ASD using CAM ranges from 28% to 95% (Akins et al., 2014; Hanson et al., 2007; Wong & Smith, 2006). Thereby, approximately 50% use biologically based therapy, 30% mind body therapy and 25% manipulation of body based therapy (Hanson et al., 2007). The most commonly used CAM therapies in ASD include vitamin C, omega 3 fatty acids, melatonin, acupuncture or secretin (Levy & Hyman, 2015). Diet has been suggested as a therapeutic measure for ASD symptoms and one-third of children have been treated with some dietary intervention at time of ASD diagnosis (Levy et al., 2003). A number of nutrition intervention strategies and CAM have been explored to treat behavioral symptoms in ASD, which will be discussed in more detail below.

Surveys have shown that most parents chose CAM for their child because of safety and absence of side effects (Hanson et al., 2007) and to treat core symptoms as well as comorbid disorders (e.g. attention, hyperactivity, GI symptoms, sleep) (Wong & Smith, 2006). A variety of treatment options are available for children diagnosed with ASD. Scientific evidence to support treatment of ASD is scarce and parents often choose treatment options due to anecdotal reports of other parents. Due to the heterogeneity of the symptoms, treatment plans require individualization and often involve and multidimensional approach. Because the underlying causes of ASD are not well understood, treatments often lack in specificity and focus on treating behavioral symptoms rather than the core of the disease. New data on mechanisms involved in ASD etiology will support the development of new targeted treatment options.

2.3 Genetic and Environmental Risk Factors

Evidence for the genetic contribution to ASD is based on family and twin studies (Tick et al., 2015), overlap with other genetic disorder (Rutter et al., 1994) and molecular data. Specific genetic etiology can be identified in approximately 25% of individuals diagnosed with ASD which includes single gene disorders (Fragile X syndrome), genetic syndromes, chromosomal anomalies, *de novo* and inherited copy number variation and single nucleotide variation (Betancur, 2011; Iossifov et al., 2012). *De novo* mutations can lead to loss of function, single-nucleotide variants or insertion/deletion variants and contribute to ASD symptoms. Common alleles that contribute to the development of ASD are difficult to identify due to the heterogeneity of the disorder and no strong contribution has been identified yet (State, 2010). Instead studies have discovered rare variants, especially copy number variants (CNVs) and sequence variants, that confer susceptibility. Susceptibility genes have been identified in varying

chromosomal regions by whole genome sequencing studies. Gene mutations in the γ-amino butyric acid (GABA) receptor subunits genes and genes have been proposed to be involved in the serotoninergic system (Klauck, 2006). To date, up to 100 genes have been discussed to be involved in ASD; however, their association is often indirect and no single gene has been specifically identified for ASD. Other genetic variations associated with ASD include variations in genes encoding chromatin remodeling, transcription and splicing and synaptic function (De Rubeis et al., 2014), presynaptic vesicle cycling and transport, cytoskeleton dynamics, cell adhesion, translational regulation, protein turnover and degradation. Because of the vast variety in genetic abnormalities, an overview of genetic abnormalities in ASD are available from different sources including Autism Chromosome Rearrangement Database (http://projects.tcag.ca/autism), SFARI gene (https://://gene.sfari.org/autdb/Welcome/do) and Autism Genetic Database (http://wren.bcf.ku.edu/).

Environmental Risk Factors: Because of the variance in ASD risk as well as the difference in symptom severity among monozygotic twins, environmental risk factors have been estimated to account to up to 55% of ASD diagnoses (Hallmeyer er al., 2011). These risk factors could influence neurodevelopment at different stages pre- or postnatally, which are critical periods in neurodevelopment. Especially the perinatal period has been heavily investigated as prenatal insults can lead to long-lasting disturbances in the developing fetus. In ASD, a combination of environmental factors is likely to be involved in the development of symptoms (Gardener et al., 2009). Thereby, critical periods have been identified in which an environment exposure is most likely to demonstrate an association with ASD development (Lyall et al., 2014).

Prenatal and perinatal environmental exposures indicated to increase the risk of ASD include dietary factors, maternal diabetes, stress, medications or viral or bacterial infection

(Herbert, 2010). Proposed dietary risk factors include maternal folate and iron status or polyunsaturated fatty acid (PUFA) intake (Schmidt et al., 2014; Lyall et al.; 2013, DeVilbiss et al., 2015). Supplemental folic acid and adequate PUFA intake are associated with decreased risk of ASD (Schmidt et al., 2012; Suren et al., 2013; Lyall et al., 2013). Likewise, maternal dietary intervention in a rodent model could ameliorate some behavioral deficits observed in the offspring caused by a prenatal insult (Kang, Kurti, et al. 2014).

Maternal obesity during pregnancy could increase the risk of the newborn for developing key features of ASD, including decreased social interaction (Li, Ou, et al., 2016; Krakowiak et al., 2012). Inconsistent evidence is presented in the literature regarding smoking and alcohol exposure and the risk of ASD. Whereas some studies report no association between smoking or alcohol and risk for ASD (Lee et al., 2012), others document a positive correlation (Ronald et al., 2010). Air pollution and endocrine disrupting chemicals (e.g. phthalates) show elevated risk for developing ASD (Lyall et al., 2014). Prenatal exposure to sodium valproate poses a significant increase in the risk of ASD diagnosis (Christensen et al., 2013). A meta-analysis including prenatal risk factors besides maternal nutrition status found an increased risk of developing ASD symptoms with gestational diabetes, maternal bleeding during pregnancy, psychiatric medication during pregnancy, advanced maternal and paternal age and birth order. When controlling for covariate, the effect was also significant with intra-taurine infections and nausea/vomiting (Gardener et al., 2009). Antibiotic use, hospitalization, duration of breastfeeding and route of birth are associated with increased incidence of ASD (Niehus & Lord, 2006; Curran et al., 2015; Schultz et al., 2006).

Additional evidence for maternal infection during pregnancy as a risk factor for developing ASD stems from animal models of maternal immune activation. Maternal immune

response to viral infection can alter brain development and lead to deficits in social interaction (Shi et al., 2003). Additionally, ASD-like behavior is observed in a model of maternal immune activation in mice (Malkova et al., 2012). It has also been hypothesized that ASD might be caused by an autoimmune reaction in the brain triggered by a previous maternal viral infection (Singh, 2009).

Immune system disturbances are widely reported in children with ASD. Thus, a few studies investigated postnatal infection as a risk factor for ASD. In a Danish cohort study investigating the association between hospitalization during childhood and diagnosis with ASD children hospitalized for an infection were more likely to be diagnosed with an ASD compared to children who were never hospitalized and a stronger associated was observed with increased number of admissions, but the authors did not find a causal relationship (Atladóttir et al., 2010). On the other hand, an earlier study did not find an association between the rate of infection and later ASD diagnosis in the first 2 years of life, but a modest increase in the first 30 days compared to controls (Rosen et al., 2007). Mechanisms underlying the development of ASD through environmental exposures are believed to involve gene-environment interactions and epigenetic changes.

Gene-environment interaction and epigenetic modification: The heterogeneity of ASD phenotypes might not only be explained by the variety in genetics and differences in environmental exposures, but also by the combination of these factors. Specifically, during the prenatal period, exposure to environmental factors could trigger the development of ASD symptoms in genetically susceptible individuals. Especially *de novo* changes in DNA described above might be indicative of gene-environment interaction. Offspring in a rodent model with a high genetic susceptibility to ASD show more severe ASD-like symptoms when exposed to a

maternal infection compared to a conventional strain (Schwartzer et al., 2013). To date, most of the human research on epigenetic modification in ASD focuses on DNA methylation as well as the dietary factors and the enzymes involved in this process.

Interaction between genetics and the environment in ASD was first suggested to prenatal complications. In an epidemiological study, children with ASD had more prenatal complications compared to their unaffected siblings (Glasson et al., 2004). Altered levels of DNA methylation was found in brain tissues of individuals with ASD in genes that are important during brain development (Nardone et al., 2014). It has been hypothesized that an early prenatal immunological insult contributes to DNA methylation and subsequently development of ASD symptoms (Nardone et al., 2014). Additionally, interactive effects were found between copy number variations and maternal infection in individuals presenting with severe social communication impairments and repetitive/restricted behaviors, suggesting an interaction between genetic susceptibility and environmental insult in ASD development (Mazina et al., 2014). The maternal nutritional status, especially levels of folate and methionine, is suggested as the one of the most significant factor causing DNA hypomethylation in the offspring with ASD. Not only the nutritional status of the mother during pregnancy, but also the concentration of methyl cycle substrates and co-factors in the children might play an important role in epigenetic modifications and subsequent behavioral changes. For example, decreased blood concentrations of vitamin B12, folate and methionine have been reported in children with ASD (Schaevitz et al., 2012). Additionally, gestational diabetes and smoking during pregnancy, two factors known to be associated with ASD, have been found to cause dysregulation in DNA methylation (Finer et al., 2015; Lee et al., 2015).

Although increased awareness, improved detection and broadening of the ASD diagnostic criteria contribute to the rise in ASD diagnoses observed in the past decade, other factors are believed to account for up to half of new cases (Hertz-Picciotto & Delwiche, 2009; King & Bearman, 2009). Identifying these factors is difficult but would allow for more meaningful family counseling, medical monitoring and treatment (Battaglia & Carey, 2006).

2.4 Intestinal Environment in ASD

Development of the GI Microbiota from in utero to Adulthood: The word microbiota is defined as a collection of microbes, including bacteria, viruses and fungi. The human GI tract is predominantly inhabited by bacterial species and contains approximately ~3 million bacterial genes or approximately 100-times as many genes as the human genome (Human Microbiome Project Consortium, 2012).

Intestinal colonization is a successive process throughout the course of life and is influenced by genetic factors, mode of delivery (vaginal birth vs. Cesarean section), early feeding practices (breast feeding vs. formula feeding), gestational age and birth environment (Penders et al., 2006). Some bacteria that become part of the adult microbiota already colonize the gut during the first months of life (Jost et al., 2015). The GI microbiota is very diverse between newborns but converges to a more generalized profile with age (Bergström et al., 2014). Bacterial species vary significantly among the infant population and can appear and disappear transiently. Low species diversity and high instability are characteristic within the first year of life and large compositional shift in abundances of major bacterial taxa appear over time (Palmer et al., 2007). Early colonizers are usually facultative anaerobes (e.g. Enterobacteria or *Lactobacillus*), followed by anaerobic genera including *Bifidobacterium*, Bacteroides or *Clostridium*.

The notion that the infant gut is sterile at birth has recently been challenged by studies revealing that the infant is exposed to microbiota *in utero*. Detection of a microbiome in the meconium of newborn infants suggests intrauterine colonization on the neonatal gut (Rautava et al., 2012). Previously the presence of bacteria in the placenta and amniotic fluid has long been associated with preterm birth, but recent reports suggest that *in utero* bacteria colonization in nonpathological situations might also influence prenatal programming (Collado et al., 2016; Rautava et al., 2012; Jiménez et al., 2008). A unique low-abundance, low richness and low diversity microbiome is present in the placenta and amniotic fluid, with Proteobacteria as the major phylum and other commensal microbiota from Firmicutes, Tenericutes, Bacteroidetes and Fusobacteria phyla (Aagaard et al., 2014; Collado et al., 2016). On the genus level *Propionibacterium, Streptococcus* and *Bifidobacterium* were found in placenta samples (Collado et al., 2016; Satokari et al., 2009). On the species level, *Lactobacillus rhmnosus* was identified in placenta samples (Satokari et al., 2009). Likewise, *Enterococcus faecium*, *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Streptococcus sanguinis* were identified in umbilical cord blood of healthy neonates born by C-section (Jiménez et al., 2005). The exact mechanism by which microbiome colonize the placenta and amniotic fluid is unknown, but it is hypothesized that microbes could translocate from the oral cavity, vagina or maternal GI tract to the placenta and amniotic fluid (Aagaard et al., 2014). In an animal study, a genetically labeled *Enterococcus faecium* strain fed to pregnant mice was found in meconium of offspring, suggesting that microbes in fetal intestine could come from microbes present in maternal gut (Jimenez et al, 2008).

Meconium: Studies have now reported the presence of a unique meconium microbiome with high inter-individual diversity of intrauterine origin. The difference between the main taxa

detected in meconium samples and dominant groups in fecal, skin and vaginal environment of pregnant women and the detection of bacterial phyla in meconium which are also present in the amniotic fluid and placenta support the notion that microbiota in meconium is not assimilated during contact with maternal habitats during birth but during intrauterine life (Gosalbes et al., 2013; Ardissone et al., 2014). The most predominantly phyla identified in meconium samples are Firmicutes and Proteobacteria. On the genus level, bacterial populations present in meconium include *Enterococcus*, *Escherichia*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Staphylococcus*. Some species identified include *Enterococcus fecalis*, *Staphylococcus epidermis*, *Escherichia coli* and *S. aureus* (Ardissone et al., 2014; Gosalbes et al., 2013; Moles et al., 2013).

Route of birth: Upon birth neonates are exposed to the microbiota of their mother as well as the hospital environment. Naturally born infants are exposed to the mother's vaginal bacteria whereas babies born by cesarean section (C-section) are first introduced to bacteria via the mother's skin, the hospital environment or the health care workers (Dominguez-Bello et al., 2010). Overall, infants born via C-section seem to have a less diverse microbiome compared to vaginally delivered babies (Biasucci et al., 2008; Jakobsson et al., 2014). The dominant phyla in infants born via C-section Propionibacterium, Corynebacterium and Streptococcus, whereas vaginal delivered infants have higher proportion of Bacteroidetes during first 12 months (Jakobsson et al., 2014; Penders et al., 2006). On the genus level, infants born via C-section have lower abundance of *Bifidobacterium* and delayed colonization with *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, but higher levels of *Staphylococcus* and *Enterococcus* (Biasucci et al., 2008; Jakobson et al., 2014). Naturally born infants, on the other hand, have an earlier colonization of *Lactobacillus, Prevotella, Atopobium* and *Sneathia* (Hall et al., 1990;

Dominguez-Bello et al., 2010; Bäckhed et al., 2015). Lower levels of *Bacteroides fragilis* but higher abundance of *C. difficile, E. coli, Enterobacter hormaechei, E. canderogenus, Haemophilus parainfluenzae, H. aegyptius, H. influence, H. haemolyticus, Staphylococcus saprophyticus, S. lugdunensis, S. aureus, Streptococcus australis, Veilonella dispar,* and *V. paravula* were reported in C-section delivered infants whereas *B. longum* and *B. catenulatum* predominantly colonized the GI tract of vaginally born infants (Bäckhed et al., 2015).

Whether the differences in microbiome composition between vaginally and C-section delivered infants persists into later life is currently unknown. Significant differences from 4 months to up to 2 years have been reported, whereas others have not found any differences after 6 months of age (Huurre et al., 2008; Bäckhed et al., 2015). Although a gradual decrease in difference between route of birth was observed, the microbiome of C-section delivered babies remained more heterogeneous, suggesting that the mode of birth is important in shaping early microbiome (Bäckhed et al., 2015). This observation is especially important giving data that describes the long lasting effect of early microbiome composition on other aspects of health (e.g. immune system, cognition).

Breast-feeding vs. formula feeding: After birth the GI microbiota is mostly shaped by the infant's early nutrition. Human milk is the gold standard for infant nutrition and the American Academy of Pediatrics recommends exclusive breast feeding for the first 6 months of life and in combination with other foods until 12 months of age. (AAP). Human milk harbors a unique microbiome including commensal, mutualistic and probiotic bacteria of which some can be found as first colonizers of the neonatal gut (Collado et al., 2009, Solís et al., 2010). Bacteria detected in human milk include *Staphylococcus*, *Streptococcus*, *Propionibacterium* and *Enterococcus*, *Lactobacillus* and *Bifidobacterium* as well as several members of Bacteroidetes

phylum and Clostridia class (Jost et al., 2015). Additionally, human milk oligosaccharides (HMO) are resistant to digestion and can be fermented in the GI tract, thereby promoting the growth of beneficial bacteria (Jost et al., 2015; Rautava et al., 2012). Additionally, HMOs found in the mother's breast milk could predict several fecal bacterial genera (Wang et al., 2015). Even though the impact of feeding mode on microbiome composition is often contradictory, it is now generally accepted that the microbiota of breast-fed infants is dominated by Bacteroidetes and higher abundance of *Bifidobacteria* and *Lactobacilli* and lower abundance of Streptococcus and Enterococcus (Harmsen et al., 2000a; Solís et al., 2010; Wang et al., 2015). The GI microbiota of formula-infants, on the other hand, is more diverse and less stable, resembles an adult profile and is more prone to changes over time (Harmsen et al., 2000a). The microbiome in formula fed infants in generally dominated by Firmicutes and higher numbers of Bacteroides and members of Clostridia class (Harmsen et al., 2000a; Penders et al., 2006; Jost et al., 2015).

Introduction of Solid Foods: With the cessation of breastfeeding and the introduction of solid food, significant changes occur in the microbiome and a shift to an adult-like composition with higher alpha diversity and enriched in *Bacteroides*, *Bilophila*, *Roseburia*, *Clostridum* and *Anaerostipes* is observed (Bäckhed et al., 2015; Palmer et al., 2007; Koenig et al., 2011). During the shift to a more adult-like microbiome, a decrease in *Bifidobacterium* and *Lactobacillus* as well as an increase in SCFA-producing bacteria and SCFA concentrations are observed (Koenig et al., 2011; Bergström et al., 2014; Yatsunenko et al., 2012).

As the infant gets older the microbiome becomes more similar to that of the mother in composition as well as functionality, mainly to adapt to changes of energy sources (Bäckhed et al., 2015). Thereby, the initial microbial exposure is important in shaping the successional trajectories leading to more complex and stable adult ecosystem (Biasucci et al., 2008).

Although previous studies have suggested that the GI microbiota becomes relatively stable and resembles that of an adult after the first 3 years of life, (Palmer et al., 2007, Bergström et al., 2014 Yatsunenko et al., 2012), other studies suggest that the GI microbiota might have a prolonged development into pre-adolescence and profound differences in microbial composition and functional potential exist between children and adults (Hollister et al., 2015). Most notably, lower abundances of Bacteroidetes but greater abundances of Firmicutes and Actinobacteria were identified in children's microbiome compared to adults (Hollister et al., 2015; Agans et al., 2011, Ringel-Kulka et al., 2013). Additionally, differences in abundances of Bacilli, Clostridium cluster IV and Bifidobacterium were observed (Agans et al., 2011; Ringel-Kulka et al., 2013). Besides compositional changes, functional changes occur as the as the GI microbiota matures to a more adult-like composition (Yatsunenko et al., 2012). For example, whereas the earlier microbiome is specialized for lactate utilization and developmental functions (e.g. folate metabolism), bacterial species at later time points are more specialized for plant polysaccharide utilization, vitamin biosynthesis or xenobiotic degradation (Koenig et al., 2011).

Other influences that could change the microbiota composition in its development are the mother's diet, antibiotic use and gestational age. Antibiotic use was linked to a decrease in *Bifidobacteria*, *Lactobacilli* and *Bacteroides* (Penders et al., 2006). Infants born preterm or late preterm presented higher counts of *C. difficile* and lower levels of coliform organisms as well as *Lactobacilli* (Penders et al., 2006, Hall et al., 1990). Additionally, differences in diversity between different geographical regions as well as temporal variability at the genus level are common during the microbiome development (Lin et al., 2013, De Filippo et al., 2010; Yatsunenko et al., 2012). Childhood may provide opportunities for microbiome interventions to promote health.

Differences in Microbial cComposition between Children with ASD and Unaffected Controls

Microbial dysbiosis has gained increasing attention in recent years as a potential environmental factor contributing to ASD symptomology via the microbiota-gut-brain axis (Bienenstock et al., 2015). In 1997, Bolte first hypothesized that *C. tetani* might be involved in ASD symptomology (Bolte, 1998). Since then, a number of human studies have evaluated the intestinal microbiota in children with ASD showing differences on the bacterial phyla and genus levels. Additionally, germ-free mice exhibit significant social impairments, suggesting that the GI microbiota could contribute to some symptoms of ASD (Desbonnet et al., 2014). Several studies have shown changes in the relative abundances of Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia (Finegold et al., 2010; Kang et al., 2013; De Angelis et al., 2013). Specifically, higher abundance of Bacteroidetes and Proteobacteria, but lower abundance of Firmicutes and Actinobacteria was observed in a subset of children with ASD. Additional studies have revealed bacterial differences at the genus level, including significant reductions in the relative abundances of *Prevotella*, *Coprococcuss*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Staphylococcus*, *Ruminococcus* and *Bifidobacteria* in children with ASD compared to non-affected controls (Kang et al., 2013; De Angelis et al., 2013; Wang et al., 2011). Conversely, *Clostridia*, *Sutterella* and *Desulfovibrio* bacteria were found to be increased in children with ASD (Finegold et al., 2010, Kang et al., 2013, Parracho et al., 2005). Significant but not consistent evidence is presented in the literature regarding microbial dysbiosis in ASD. For example, studies using sibling as the control group found no differences in the microbiota composition (Gondalia et al., 2012; Son et al., 2015). Other studies which included siblings as a control group demonstrated intermediate levels of some bacterial genera (e.g., *Clostridium*) in siblings of children with ASD (Parracho et al., 2005). These

differences could be attributed to environmental factors since siblings live in the same household as children with ASD and are exposed to the same environmental factors. Larger studies are needed to further define a potentially distinct ASD microbiome.

Besides differences in bacterial composition of the intestine in children with ASD, increasing research is investigating the composition of yeast and fungi in stool samples of children with ASD and is suspected to be involved in the gut-to-brain communication. Thus far, studies observed differences in the β-diversity of the GI mycobiota between children with ASD and controls (Strati et al., 2017). Additionally, an overall higher yeast concentration with a higher presence of *Candida spp*. was described in children with ASD compared to unaffected controls (Iovene et al., 2017; Kantarcioglu et al., 2016). Furthermore, some yeast, such as *Candida krusei* or *Candida glabrate*, that were isolated from stool samples of children with ASD stool was not found in stool specimen of control children (Kantarcioglu et al., 2016). Fungal dysbiosis has been associated with inflammation in GI disorders such as Chron's disease, but no studies have shown a correlation between GI or ASD symptoms and yeast population to date; however, it is hypothesized that higher levels of Candida can cause decreased absorption of carbohydrates and minerals and higher absorption of toxins which could contribute to some symptoms of ASD (Burrus, 2012).

In order to develop potential strategies to ameliorate some symptoms of ASD through manipulation of the GI microbiota, a few researchers are investigating associations between specific bacteria and ASD symptom severity. Reduced bacterial richness was significantly associated with ASD symptoms (Kang et al., 2013). Higher abundance of *Clostridia* and *Desulfovibrio* and lower Bacteroidetes/Firmicutes ratio was associated with ASD severity in two studies (Kang et al., 2013, Tomova et al., 2015). Delineating correlations between ASD

symptoms and microbiota is difficult due to often occurring co-morbid GI symptoms, making it difficult to decipher whether ASD symptoms and microbial dysbiosis are related to each other or to GI symptoms. For example, a decrease of beneficial bacteria was found in children with ASD and co-morbid GI symptoms (De Angelis et al., 2013, Wang et al., 2011). However, one study described the presence of ASD symptoms rather than the severity of GI symptoms was a stronger predictor for microbial changes (Kang et al., 2013). Additional evidence for specific microbiota influencing ASD symptom severity comes from probiotic supplementation studies which are described in more detail below.

Because human studies to date only allow to make conclusions about associations between the microbiota and ASD behaviors, animal models are invaluable to further investigate the role of the GI microbiota in ASD symptomology. Several mouse models using genetic mutations or prenatal exposure to environmental risk factors have been developed to investigate underlying causes for the development of ASD. Interestingly, the GI microbiota composition and microbial metabolites in these models is often different from commensal mice (De Theije et al., 2014). A mouse model that mimics prenatal insult as environmental risk factor, a dysbiotic microbiota similar to that observed in human studies of ASD with IBD was observed (Lim et al., 2017). In the same study, correlation between serum levels of serotonin and *Sporanaerobacter* was detected which let the authors to hypothesize that environmental factors might be responsible for microbial dysbiosis in ASD which in turn causes other abnormalities often found in this population such as hyperserotenemia (Lim et al., 2017). Sex-specific alterations in microbiota were observed in mouse model of ASD with *Bacteroides, Parabacteroides, Sutterella*, *Dehalobacterium* and *Oscillospira* as main drivers of the microbiota profile that was associated with changes in behavior, GI permeability and GI inflammatory state (Coretti et al.,

2017). These finding are especially intriguing due to the higher occurrence of ASD in boys compared to girls. Lastly, GI barrier defects and alterations in microbiota composition were observed in maternal immune activation (MIA) mouse model of ASD which could be corrected with oral administration of *Bacteroides fragilis*, suggesting that the GI microbiota could contribute to the development of symtpoms associated with ASD (Hsiao et al., 2013).

Bacterial Metabolites in Children with ASD

Besides differences in the microbial composition, significant deviations are also seen in the bacterial metabolites present in the feces and urine of children with ASD.

Volatile Fatty Acids: VFA (acetate, propionate, butyrate, isobutyrate, isovalerate, valerate) are the end-products of microbial fermentation in the colon and have been implicated to have various health benefits to the host (e.g. weight control, lipid profiles, colon health) (Byrne et al., 2015). VFA can be rapidly absorbed in the large intestine and be detected in the blood (Tsukahara et al., 2014). Especially during early brain development, propionate in particular serves as a major energy source for brain metabolism (Zhao et al., 2017). However, accumulation of VFA, specifically propionate, has also been shown to have broad effects on the nervous system physiology, causing developmental delay or seizures (Feliz et al., 2003). In children with ASD, elevated concentrations in stool, urine and serum have been reported and VFA-producing bacteria, e.g. Clostridia, *Desulfovibrio* and *Bacteroides* were shown to be increased in the feces (De Angelis et al., 2013; Wang et al., 2012; Adams et al., 2011a; Zhao et al., 2017). In animal studies, propionate could cause neurobiological changes similar to those observed in individuals with ASD and intraventricular administration of propionate could provoke ASD-like behavior including repetitive interests and impaired social interactions

(Thomas et al., 2012; MacFabe et al., 2011; Foley et al., 2015). The exact effect of VFA on the brain are unknown, but translocation of VFAs through the blood brain barrier (BBB) facilitated by monocarboxylic acid transporters or passive diffusion could cause potential effects on the brain and contribute to development of ASD symptoms (Conn et al., 1983). Likewise, VFAs could also interact with membrane receptors (i.e., G-coupled receptors) and trigger the activation of gene expression (Nankova et al., 2014). In addition to propionate, butyrate has been of interest in eliciting ASD-behaviors due to its similar structure to valproic acid which is used in a mouse model of ASD to induce autism-like behaviors (Nankova et al., 2014).

Serotonin: Although most serotonin, or 5-hydroxytryptamine (5-HT), is produced in the GI tract, 5-HT modulates neurodevelopment and might be important in social function and repetitive behavior (Muller et al., 2016). Elevated levels of whole blood and platelet 5-HT are consistently observed in a subset of children with ASD, making it a potential candidate as a biomarker for ASD (Gabriele et al., 2014). An adult study of acute tryptophan depletion, the precursor of central serotonin synthesis, using Magnetic Resonance Imaging showed that adults with autism had abnormal brain activation of brain regions for inhibitory control after tryptophan depletion. These brain areas might partially be related to some restrictive and repetitive symptoms observed in individuals with ASD (Daly et al., 2014).

Genetic, GI or immune changes have been proposed as potential contributing factors to the hyperserotonemia observed in children with ASD (Schain & Freedman, 1961; Anderson et al., 1987; Hanley et al., 1977); however, not all cases can be explained by genetic variations. Some bacterial strains that are known to influence 5-HT metabolism (e.g. Clostridial species, *Lactobacillus*) were increased in the stool of affected children. In patients with ASD, altered function and metabolism of neurotransmitters, such as 5-HT and catecholamines, and

dysfunction of the serotonergic system have been reported to contribute to symptomology (Muller et al., 2016; Gabriele et al., 2014; Schain & Freedman, 1961; Anderson et al, 1987; Melke et a., 2008). Evidence for aberrations in the central serotonin system in ASD come from neuroimaging of post-mortem studies showing decreased serotonin receptor binding. These results suggest that peripheral alterations in the serotonin system can affect the central serotonin signaling system (Muller et al., 2016). Interestingly, studies observed high blood but low brain serotonin levels children with ASD (McNamara et al., 2008; Zafeiriou et al., 2009). Additionally, higher serotonin levels in mothers of children have been described, indicating that the serotonin during pregnancy can cross the placenta membrane and reach the brain due to blood brain barrier being not completely formed. Due to serotonin's role in early neurodevelopment, studies have begun to investigate the potential role or selective serotonin inhibitor us during pregnancy and the increased risk for developing ASD (Harrington et al., 2013). These observations led researchers to hypothesize that hyperserotonemia occurs during neurodevelopment in children with ASD which can lead to a later loss of serotonin terminals and axons (Yang et al., 2014). Even though the mechanisms by which serotonergic signaling impact behavior in children with ASD is not completely understood, targeting serotonin neurotransmission might be a future avenue for intervention (Lacivita et al., 2017).

Other metabolites: Besides VFA and serotonin, other bacterial metabolites have been studied to a lesser extent in children with ASD. Ammonia concentrations were found to be elevated in fecal samples of children with ASD and various metabolites of bacterial metabolism of amino acids, carbohydrates and bile acids were altered, including an increase of taurocholenate sulfate and decrease in 5 amino-valerate (Wang et al., 2012, Ming et al., 2012). Differences in urinary concentrations of hippurate and phenyacetylglutamine (PAG), which

precursors are produced by bacterial metabolism, were observed (Yap 2012). 3-(3-hyrdroxy phenyl)-3-hydroxypropionic acid (HPHPA), a metabolic product of the genus *Clostridium*, was increased in urine samples of children with autism. It has been suggested that HPHPA could induce autism symptoms by depleting catecholamine concentrations in the brain (Keşli et al., 2014). Other markers of bacterial metabolites (i.e., para-hydroxybenzoate, para-hydroxyphenylacetate, 2-hydroxyphenylacetate, 3-indoleactate, tricarballylate) were abnormal in urine of children with ASD in a different study (Esparham et al., 2015). Additionally, in an animal model of ASD, specific bacterial metabolites (4-ethylphenylsulphate; 4EPS) caused ASD-related behaviors, proposing that bacterial metabolites could be a way of communication between the GI microbiota and brain in ASD (Hsiao et al., 2013).

Gastrointestinal Symptoms in Children with ASD

In addition to abnormal microbial composition and metabolites, GI distress, such as diarrhea, constipation or abdominal pain, is prevalent among children with ASD and has been suggested to contribute to behavioral problems and to correlate with symptom severity (Tomova et al., 2015; Adams et al., 2011a; Buie et al., 2010; Coury et al., 2012a; McElhanon et al., 2014; Horvath et al., 1999). Besides GI distress, GI functional abnormalities are also observed in children with ASD. This include reduced mRNA expression of carbohydrate digestive enzymes (sucrase isomaltase and maltase glucoamylase) and hexose transporters (SGLT1 and GLUT2), which can cause carbohydrate maldigestion and malabsorption increased intestinal permeability (Williams et al., 2011). In a prevalence study with over 14,000 individuals, a significant difference in the occurrence of bowel disorders between children with ASD versus non-affected children was observed (Kohane et al., 2012). High-risk infants (infants with siblings diagnosed

with ASD) showed a greater prevalence of GI symptoms and children with ASD were more likely to report GI symptoms (e.g. constipation, food allergy/intolerance, diarrhea) in the first 3 years of life (Penn et al., 2016; Bresnahan et al., 2015). Likewise, MIA offspring displayed GI symptoms similar to symptoms observed in human ASD (Hsiao et al., 2013).

Determining an exact prevalence of GI disorders in the ASD population is challenging due to social communication difficulties in individuals with ASD and interpretation discrepancies of GI problems (de Theije et al., 2011). Likewise, the link between GI dysfunction and ASD symptoms is not well understood, but it is hypothesized to include intestinal inflammation, mitochondrial dysfunction or microbial dysbiosis. Mitochondrial dysfunction (e.g. mitochondrial enzymes or carriers) is prevalent in the ASD population and many mitochondrial diseases are associated with GI disorders (Rossignol & Bradstreet, 2008; Frye et al., 2015). Frye et al. also hypothesized that microbial dysbiosis could induce mitochondrial dysfunction and GI symptoms. For example, propionate, product of microbial fermentation, is known to influence mitochondrial metabolism and abnormalities in mitochondrial metabolism were observed in a propionate rodent model of ASD (Frye et al., 2015).

Anecdotal reports of parents claim that GI problems and behavior symptoms manifest in parallel. One study reported that the onset of GI symptoms occurred before or at time of diagnosis with ASD in 67% of cases (Williams et al., 2011). The timing of onset was also associated with levels of Clostridiales, namely Lachnospiraceae and Ruminococcacaea, suggesting that timing of onset of GI symptoms relative to onset of ASD symptoms may be associated with increases in Clostridiales (Williams et al., 2011). Additionally, in children with ASD and comorbid GI symptoms, reduced carbohydrate digestive capacity can cause carbohydrate maldigestion and malabsorption which has been associated with an increased

abundance of *Bacteroidetes*, *Firmicutes*, and *Betaproteobacteria* in the mucoepithelium (Williams et al., 2011). The resulting accumulation of nondigested carbohydrates in the intestine, in turn, could lead to intestinal inflammation and potentially contribute to ASD behavioral problems (Williams et al., 2011). Other associations between specific bacterial general and GI symtpoms were observed. Constipation was associated with higher levels of *Escherichia/Shigella* and Clostridium Cluster XVIII (Strati et al., 2017) and *Sutterella* was mostly increased in children with GI symptoms but not in those without (Williams et al., 2012). *C. perfringens* and the toxin-producing genes were found in higher abundances in children with ASD and co-morbid GI symptoms (Finegold et al., 2017). Although in non-ASD patients, changes in the intestinal microbiota are implicated to contribute to the development of GI disorders, Gondalia et al. (2012) found no differences in microbiota composition between children with ASD with or without GI dysfunction (Gondalia et al., 2012).

Increased inflammatory responses and immune dysregulation might also play a part in the increased prevalence of GI symptoms in children with ASD. In a subset of children with ASD, inflammatory mucosal pathology, increased T-cell activation, increased cytokines and immunoglobulins and histological changes were found in in intestinal biopsies of children with ASD and co-morbid GI symptoms (Ashwood & Wakefield, 2006). In the peripheral blood and intestinal mucosa increased pro-inflammatory cytokines, increased TNFα, IFNγ, IL-4 and IL-5 similar to levels found in neurotypical children with Chron's Disease were found in children with ASD (Ashwood & Wakefield, 2006). Thereby, the inflammatory status might also be related to abnormal microbial profile. Luna et al (2017) found a unique mucosa-associated microbiome with an increased abundance of Clostridiales but a decrease in *Dorea*, *Blautia* and *Sutterella* in children with ASD and GI disorders that was also correlated with peripheral cytokine and

tryptophan levels. (Luna et al., 2017). The group identified relationships between microbial taxa, serotonin metabolites and inflammatory cytokines that could suggest a potential interconnection between GI symptoms, GI microbiota and peripheral immune or metabolic markers (Luna et al., 2017).

2.5 Nutrition and ASD

Feeding Problems in Children with ASD

Achieving adequate nutritional intake presents a big challenge in children with ASD due to GI symptoms, food allergies, metabolic abnormalities or problematic eating behaviors. Approximately 90% of children with ASD experience some sort of feeding-related concern and often present as multidimensional problems (Ledford & Gast, 2006, Nadon et al., 2011). In fact, the major reasons for referral of children with ASD to a Dietitian are concerns about food selectivity and dietary adequacy (Bowers, 2002). However, some eating problems might be able to be restored as older children with ASD tend to have less eating problems compared to younger children with ASD, potentially due to behavioral therapies (Laud et al., 2009).

Evidence for an association between food selectivity and ASD symptom severity is not conclusive. Whereas some studies suggest that food selectivity and feeding problems were associated with higher rates of ASD symptoms as well as severity of temper tantrums (Crasta et al., 2014; Postorino et al., 2015; Dominick et al., 2007), other studies found no association between feeding difficulties and severity of social, communication and cognitive deficits (Johnson et al., 2014). It has been suggested that severity of ASD symptoms might related to topography and duration of refusal behavior but not necessarily directly to food selectivity (Aponte & Romanczyk, 2016). Some of these discrepancies could be due to different definitions

of food selectivity used in the various studies as well as the methodology used to assess feeding problem behaviors (Postorino et al., 2015). For example, food selectivity can refer to picky eating, food refusal, limited food repertoires, excessive intake of a few foods and selective intake of certain food categories (Cermak et al., 2010).

Picky eating, food refusal and food selectivity are commonly reported problematic eating behaviors and some children with ASD might eat as little as five foods (Cermak et al., 2010). Thereby, food selectivity is usually based on color, shape, texture or temperature of the food (Schreck et al., 2004). Likewise, children with ASD might require specific utensils, a particular food presentation or sitting at specific places at the table and tactile and oral defensiveness may result in difficulty with textures (Schreck et al., 2004; Cermak et al., 2010, Johnson et al., 2014). Picky eating behaviors might be a manifestation of repetitive behavior patterns, ritualistic or externalizing behaviors (Johnson et al., 2014). Others suggest that picky eating might be a reflection of the child's resistance to change, inflexibility, sensory sensitivities, inadvertent reinforcement of negative mealtime behaviors, GI problems and oral motor delay (Johnson et al., 2014, Cermak et al., 2010). Sensory sensitivities which are very common among children with ASD can negatively affect eating behaviors. However, more research is required to determine whether other factors such as parental preferences and family mealtimes can contribute to food selectivity in children with ASD (Cermak et al., 2010). Family mealtimes might especially be important as disruptive behaviors often seen in children with ASD can cause more stressful meals (Johnson et l., 2014). Additionally, parent behavior could model specific restricted diets and expose the child to a limited range of foods (Aponte & Romanczyk, 2016). Using regression analysis, one study found that family food preferences instead of ASD symptom severity was the strongest predictor of child's food preferences (Schreck et al., 2004).

Besides food selectivity other prevalent behavioral problems at mealtimes in children with ASD include eating fast, difficulty processing table foods, gagging/coughing on textures, not feeding self, taking long time to eat meals, not communicating when hungry, stressful and prolonged meal times and eating inadequate amounts (Bicer & Alsaffar, 2013; Malhi et al., 2017). Likewise, children with ASD are more likely to present with food allergies and parental report of allergies include milk/dairy, nuts and fruits (Lyall et al., 2015; Gurney et al., 2006). Several studies have found that children with ASD eat fewer foods overall and a strong preference for starches, snack and processed foods, while rejecting fruits, vegetables and or protein is common in ASD (Bicer & Alsaffar, 2013; Al-Farsi et al., 2011, Malhi et al., 2017). Comparing fruits and vegetables intake of children with ASD to healthy controls showed that daily servings of fruits and vegetables were significantly lower in children with ASD (Marí-Bauset et al., 2013; Schreck et al., 2004). The restrictive eating patterns and difficulties in other aspects of a healthy lifestyle (e.g. physical activity) due to impaired social interaction and communication, can lead to the development of malnutrition and research has reported higher rates of underweight, overweight and obesity in children with ASD compared to unaffected children (Bicer & Alsaffar, 2013; Sharp et al., 2013; Curtin et al., 2010). Obesity related complications (e.g. hypertension, diabetes) are also more prevalent among adults with ASD (Berry et al., 2015).

Interestingly, eating problems from birth can be observed in 20-30% of children who were later diagnosed with ASD (Dominick et al., 2007). One study reported that overall children with a later diagnosis of ASD had more consultations for feeding problems in early life compared to children who did not get diagnosed (Olsson et al., 2013). Common early feeding problems observed are late acceptance of solid foods and slow feeding in early years of life

(Edmond et al., 2010). Additionally, at 15 months of age diet of children later diagnosed with ASD were less varied than diets of typically developing children and the variety became progressively less with age (Edmond et al., 2010). Feeding problems early in life are especially of concern as feeding problems in childhood have been associated with poor growth, developmental outcomes and unwanted medical procedures (Johnson et al., 2014).

Nutrient Deficiencies

The restricted food intake, ritualized eating habits and problematic mealtime behaviors paired with malabsorption issues can have an impact on the nutritional status of children with ASD (Liu X, et al., 2016). Additionally, parents often adopt diets which anecdotally improved the behavior of some children without the guidance of a nutrition specialist which could put the kids at further risk of developing nutrient deficiencies. For example, following a gluten-free/casein-free diet can put children at risk for insufficient intake of calcium because of eliminating dairy products from the diet. The impact on the nutritional status can be seen in intakes lower than the recommendation as well as nutrient deficiencies which can be detected in the blood of children with ASD. However, conflicting results can be found in the literature and studies are indicating that children with ASD have intakes above, below or same as children without ASD (Cermak, et al., 2010). Thereby, the lack of studies in comparing children with and without food selectivity, making it is difficult to determine whether food selectivity places children with ASD at risk for malnutrition.

Micronutrients are important for neural development and brain functioning (Sandstead, 2000). Thus, it has been suggested that micronutrient deficiencies could contribute to ASD pathophysiology. In general, the total mean average of nutrient deficiencies is higher in children

with ASD compared to unaffected controls (Zimmer et al., 2012; Shmaya et al., 2015) and selective eaters among the ASD population are more likely to have inadequate intake in at least one nutrient (Zimmer et al., 2012). Case reports of children with ASD who were at risk for developing scurvy, rickets or ophthalmological conditions due to unrecognized nutrient deficiencies have been published (Niwa et al., 2012; Cole et al., 2011; Clark et al., 1993). In one of the case reports the child's intake was limited to French Fries and water for several years which caused the development of vitamin A and vitamin D deficiencies (Clark et al., 1993). Decreased levels in whole blood, serum or plasma levels were found for pantothenic acid, folate, biotin, vitamin B12, vitamin D and vitamin E (Liu X, et al, 2016; Ranjan & Nasser, 2015; Zimmer et al., 2012). Magnesium, iodine, iron and chromium and selenium were found in lower concentrations in children with ASD compared to non-affected controls (Ranjan & Nasser, 2015). Likewise, circulating levels of calcium were found in lower concentrations in children with ASD (Bicer & Alsaffar, 2013; Ranjan & Nasser, 2015; Zimmer et al., 2012) which is of concern due to the importance of calcium in supporting bone growth. Some nutrients might correlate with severity of ASD symptoms, for example, vitamin A or folate intake (Liu X, et al., 2016). Interestingly, gender differences were observed in the blood levels of vitamin D levels in children with ASD with levels being lower in young males compared to females (Kočovská et al., 2014).

Compared to the effect of feeding problems in the ASD population on micronutrient intakes and concentration, evidence for macronutrient inadequacies are less prominent. One study observed a decreased intake of macronutrients in children with ASD compared to healthy controls (Liu X, et al., 2016; Zimmer et al., 2012), whereas no difference in energy intake was observed in other studies (Malhi et al., 2017; Emond et al., 2010). Lastly, approximately 70% of

children with ASD do not meet the recommendations for dietary fiber intake (Bicer & Alsaffar, 2013) which can have a profound effect on microbial diversity.

Fatty acids have been of increasing interest to ASD related research. For example, Docosahexaenoic acid (DHA) is important component of neural development, in maintenance of neural plasticity neuronal signaling and neurotransmitter uptake (Wainwright, 2002; Dufault et al., 2009). Concentrations of omega-3 fatty acids (DHA, APA) are lower in ASD children whereas omega-6 fatty acid levels in the plasma were reported to be higher (Ranjan & Nasser, 2015; Esparham et al., 2015). Analysis of red blood cell phospholipids in children with ASD aged 3-17 years revealed a decrease of arachidonic acid (AA) and DHA and an increase in pro-inflammatory prostaglandin E2 (Brigandi et al., 2015). Additionally, supplementation with omega-3 fatty acids in human and animal models might be efficacious in alleviating some symptoms of ASD, suggesting that omega-3 fatty acids could play a role in the etiology of ASD. Potential mechanisms explaining the abnormal levels of fatty acids in children with ASD include dysfunctional lipid metabolism, abnormalities in maintenance of phospholipid membranes or elevation of lipid peroxidation (Tamiji & Crawford, 2010).

Dietary Interventions

Due to the lack of effective medical treatment approaches, parents often seek alternative treatment options for their children after ASD diagnosis. Although there is often no convincing justification for the efficacy of alternative treatments, dietary changes that are considered risk free by the lay public are often adopted (Stewart et al., 2015). Animal and human studies have provided evidence for a potential dietary intervention in alleviating some symptoms of ASD. It has been estimated that one-third of children have been treated with some dietary intervention

after of ASD diagnosis (Levy et al., 2003). An expert panel has set guidelines for the nutritional management of GI symptoms in children with ASD (Berry et al., 2015). Specific considerations have to be taken into account when working with children with ASD because of the high prevalence of food selectivity and general feeding problems. For example, if the child suffers from constipation the types of fruits, vegetables and whole grains that the child is accepting in the diet need to be evaluated (Berry et al., 2015). A number of nutrition intervention strategies including gluten-free, casein-free diet (GFCF) or supplementation with omega-3 fatty acids, minerals or multivitamins have been explored to treat behavioral symptoms and comorbid GI distress. Some diets (e.g. GFCF, antioxidant diet or ketogenic diets) have shown an alleviation of behavior and GI symptoms associated with ASD, whereas others saw no behavioral differences compared to controls or even reported insufficient or excessive nutrient intake in response to interventions (Millward et al., 2008; Mousain Bosc, et al., 2006; Srinivasan et al., 2009; Knivsberg et al., 2002).

Gluten-free/Casein-free diets: One of the most studied dietary interventions in children with ASD is the GFCF diet. The rationale behind the GCFC diet as a therapeutic intervention for children with ASD is the opioid-excess theory. This theory is based on the limited ability of children with ASD to break down gluten and casein coupled with the leaky gut theory which allows the peptides to translocate into periphery. In the CNS, the peptides can bind to opioid receptors and impact neural development, cognitive function, attention, learning and behavioral symptoms associated with ASD (Whiteley et al., 1999). Support to this theory stems from studies reporting increased antibodies against casein, gluten and gliadin (Vojdani et al., 2003). GFCF intervention studies have yielded mixed results. Knivsberg et al. (2002) demonstrated that children with ASD on a GFCF diet showed significant improvements in social interaction,

communication and cognition compared to children with ASD not following a specialty diet and these improvements in autistic behaviors persisted in a one-year follow-up assessment (Knivsberg et al., 2002). In another randomized control trial, 12 months GFCF diet intervention improved in domains of social interaction and repetitive behaviors in children with ASD aged 4-10 years (Whiteley et al., 2010). On the other hand, no improvement in behavior after GFCF intervention was observed in other studies (Elder et al., 2006; Johnson et al., 2011; Hyman et al., 2016). In a 12-week intervention study in children age 2-16 years, no changes in behavioral and urinary metabolic outcomes were observed (Elder et al., 2006). Because elimination diets, including GFCF, can put children at risk for nutrient deficiencies, the use of such diets should be evaluated on a case-to-case basis and elimination of foods should only be implemented if either an allergy or intolerance to food allergens has been diagnosed or other GI symptoms due to food allergens are present in the child (Marí -Bauset et al., 2016; Lange et al., 2015). Additionally, the evidence present to date is weak because of diverse methodology between studies and the lack of an appropriate control group in some studies (Lange et al., 2015).

Other Diets: Other dietary interventions that have been explored, including FODMAP (Fermentable Oligo-Di-Monosaccarides and Polyols), elimination diets, ketogenic diet, avoidance of food coloring/food additives or specific carbohydrate diet, show some improvements, but the evidence to support specialty diet as a therapeutic measurement is inconclusive (Berry et al., 2015). The **t**heory behind ketogenic diet as a therapeutic measure for symptoms of ASD is the proposed mitochondrial dysfunction that can disrupt synaptic functioning and neuronal integrity in individuals with ASD. In a rodent model of ASD, adherence to a ketogenic diet improved social behaviors and mitochondrial metabolism (Ahn et al., 2014). Interestingly, in another animal study the ketogenic diet improved sociability and

reduced repetitive behavior in female mice, but only had limited effects in male mice (Ruskin et al., 2017). Human studies on the ketogenic diet also showed some improvement in social communication and other ASD-related behaviors (Evangeliou et al., 2003). In a pilot study of 6-month diet intervention using the ketogenic diet, some improvement of ASD symptoms were observed in subjects with milder symptom severity (Evangeliou et al., 2003).

Nutrition supplements: Nutrition supplements are also commonly used among the ASD population; the most commonly used supplements among the ASD population are multivitamin/mineral supplements (Stewart et al., 2015). However, most multivitamin/mineral supplements do not provide adequate amounts of micronutrients that children in ASD are typically deficient in (e.g. choline, potassium) (Stewart et al., 2015). Supplementation studies with specific micronutrients produced promising results. Because low levels of vitamin D in early childhood have been hypothesized to be an environmental risk factor for developing ASD, children with ASD often have low vitamin D intakes and blood levels of vitamin D have been found to be lower in children with ASD, vitamin D supplementation studies have gained increasing interest as a potential therapeutic option. Some vitamin D intervention studies show promising results (Mazahery et al., 2016). Additionally, vitamin D is neuroprotective through immunomodulatory properties, reduction of inflammatory cytokines and interaction with neurotransmitters (McGrath et al., 2001; Kalueff & Tuohimaa, 2007). In an animal model of ASD, vitamin D exhibited protective effects and ameliorated neurochemical and inflammatory aberrations associated with ASD-like behaviors (Alfawaz et al., 2014). On the other hand, in a randomized placebo-controlled trial with vitamin D supplementation for 20 weeks no effect on stereotypical behavior was observed (Kerley et al., 2017). Other vitamins that have been investigated in relation to lessening some symptoms of ASD vitamin B_6 and vitamin C which

showed improvement in some behaviors after supplementation (Martineau et al., 1985; Dolske et al., 1993). Lastly, in a large trial of 141 children a vitamin/mineral multi-supplement improved the nutritional and metabolic status of children with ASD as well as some behaviors associated with ASD (Adams et al., 2011).

Fatty acid supplementation: Most of the brain's dry weight is composed of fat with 40% of polyunsaturated fatty acids (PUFAs) in the brain with an omega-3 fatty acid, docosahexaenoic acid (DHA) (Mazahery et al., 2017). Due to its high concentrations, the important role of PUFAs in supporting normal neurodevelopment and function as been recognized (Bourre et al., 1991) and the efficacy of fatty acid supplementation in managing some symptoms of ASD has gained an considerable amount of attention. Likewise, aberrations in lipid metabolism and deficiencies of some beneficial PUFAs have been reported in individuals with ASD (Ranjan & Nasser, 2015; Esparham et al., 2015). Rodents with autism-like behavior induced by valporic acid exposure supplemented with gamma-linolenic (GLA) acid were more protected from neuronal degeneration and loss compared to with alpha-linolenic acid (ALA) (Yadav et al., 2017). A recent meta-analysis including studies on omega-3 fatty acid supplementation found that omega-3 supplementation improved autism-associated behaviors such as hyperactivity, lethargy and stereotypy but found no difference in social responsiveness (Cheng et al., 2017). A different meta-analysis concluded that omega-3 fatty acid supplementation improved social interaction and repetitive behaviors compared to placebo controls (Mazahery et al., 2017). The authors report that six open-label trials resulted in beneficial effects of PUFA supplementation whereas four randomized controlled trial produced inconclusive evidence (Mazahery et al., 2017). Additionally, a additive beneficial effect of supplementing vitamin E with omega-3 fatty acids

was reported due to the protective role of vitamin E against oxidative damage (Gumpricht & Rockway, 2013).

Dietary interventions to target the GI *microbiota:* A healthy, nutrient-rich diet has been proven to support good mental health (O'Neil et al., 2013). Due to the profound effect of diet on the GI microbiota composition and the newly acquired knowledge about the gut-brain-connection, the GI microbiota was proposed to be key mediator in the diet-brain health connection (Dawson et al., 2016). The connection between diet and brain mediated through the microbiota was demonstrated in a 4-week administration of a 125g-fermented milk product containing *Bifidobacterium animalis subsp Lactis* CNCM I-2494, *Streptococcus thermophiles* CNCM I-1630, *Lactobacillus bulgaricus* CNCM I-1632 and I-1519 and *Lactobacillus lactis subsp Lactis* CNCM I-1631 to healthy women which affected activity of brain regions that control processing of emotions and sensation. Additionally, consumption of 1.5 g/day of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 by healthy men and women alleviated general signs of anxiety and depression. (Tillisch et al., 2013). Furthermore, highly processed foods can decrease microbial diversity and were linked to increased risk for mental disorder (Dawson et al., 2016). In an animal model, rodent maintained on a lean beef supplemented diet for 3 months showed marked diet-induced shifts in the GI microbiota diversity as well as improved reference and working memory and decreased anxiety-like behavior (Li et al., 2009). Due to the increasing knowledge in how GI microbes could potentially influence behavior and reports of microbial dysbiosis in children with ASD, dietary interventions to manipulate the GI microbiota to ameliorate some symptoms of ASD could be a promising therapeutic avenue. As previously described dietary interventions improved some behaviors in children with ASD, leading researchers to investigate the potential impact of these dietary

interventions on the GI microbiota compositions as a potential links. Likewise, a decrease in carbohydrate-fermenting bacteria in some studies suggests that dietary patterns could potential impact GI microbiota composition and behavior in children with ASD (Kang et al., 2013). In a murine model of ASD, ketogenic diet triggered a reduction of microbial abundance and reversed some microbial profiles seen in children with ASD, such as low Firmicutes/Bacteroidetes ratio, increased abundance of *Akkermansia muciniphila* and SCFA producing Clostridia species (*C. coccoides* and *C. leptum* (Newell et al., 2016). Lastly, the purpose of the specific carbohydrate diet, which is used by some parents to manage symptoms of ASD, is to alleviate symptoms of malabsorption and prevent growth of potentially pathogenic bacteria which could potentially affect symptomology (Gottschall, 2004).

In summary, a multidisciplinary approach to working with a child with ASD and feeding problems is warranted. For example, occupational therapist can work on improving feeding behavior (i.e., by a sensory integration approach), dietitians can identify nutritionally adequate diets and psychologist can use behavioral approaches to increase child's acceptance of foods (Cermak et al., 2010). Most studies on dietary supplementation have small sample numbers making it difficult to draw definite conclusions. Future larger randomized controlled clinical trials are needed to investigate the efficacy of diet as a therapeutic measure to management some symptoms of autism.

Pre- and Probiotics use: Because of the health benefits commonly reported by pro- and prebiotics intake and new data suggesting a role of the microbiota in ASD symptomology, some clinical studies have explored the effect of probiotic supplementation on ASD symptoms and its use is highly discussed in the literature (**Table 1.1**). Some improvement of symptoms as well as microbial and metabolite imbalances were observed in children with ASD and animal models

after probiotic treatment (Tomova et al., 2015; Hsiao et al., 2013, Kałużna-Czaplińska & Błaszczyk, 2012; West et al., 2013; Russo, 2015). For example, children with ASD taking a probiotic mixture (*Lactobacillus acidophilus, L. casei, L. delbruecki, Bifidobacterium longum, B. bifidum*) with an immunomodulatory three times daily for 6 months, had improved GI function had decreased ASD symptom severity as well as an improvement of GI symptoms. Recently, the prebiotic B-GOS showed a positive modulation of the microbial community in an *in vitro* model using fecal inoculum of children with ASD (Grimaldi et al., 2017). Additionally, in an animal model of ASD amelioration of ASD-like symptoms after supplementation with *B. fragilis* were observed (Hsiao et al., 2013). Because of significant differences in the dose and intervention length between studies, it is difficult to provide definite recommendations on the amount and kind of probiotic to use. In addition, no dietary information was collected and not all groups analyzed microbiota changes after the probiotic treatment or included a control cohort. However, according to a physician's survey, is recommended by one fifth of physicians in treating ASD symptoms (Critchfield et al., 2011; Golnik & Ireland, 2009).

A new approach to manipulate the GI microbiota in order to manage some symptoms of ASD is the Fecal Microbial Transfer (FMT). FMT has been shown to be very efficacious in treating *C. difficile* infections in elderly patients. In an open-label study investigating the possibility of FMT in children with ASD showed improvements in GI symptoms, parents' perceived behavior, overall severity of symptoms, social impairments as well as ASD-associated behaviors such as irritability, lethargy, stereotypy, hyperactivity and inappropriate speech (Kang et al., 2017).

2.6 The Microbiota-Gut- Brain Axis

The microbiota-gut-brain axis has been described as a way of communication between the gut microbiota and the brain. The symbiotic relationship between GI microorganisms and its host has long been described and evidence for the involvement of the GI microbiota in critical body processes such as providing protection against pathogens, regulation and programming of the immune system and metabolism has been established (Forsythe et al., 2015; Kabat et al., 2014; O'Hara & Shanahan, 2006). In recent years, emerging research highlighted the role of the microbiota in regulating the Central Nervous System (CNS) and influencing cognitive function and behavior. Germ-free (GF) and gnotobiotic (GN) animal models have been fundamental in establishing the connection between the GI microbiota and behavior. GF mice show a behavioral profile that is characterized by alterations in anxiety-like behavior, locomotor behavior, learning and memory, social cognition and social preference (Luczynski et al., 2016). On the cellular and molecular levels, GF mice exhibit aberrations in neuronal activity, neuroprotection (e.g. increased permeability of the blood brain barriers), expression of neurotransmitters and receptors and stress hormone signaling (Luczyncksi et al., 2016). Similarly, bacterial genera might also be associated with brain structure (Fernandez-Real et al., 2015). How changes in microbial composition relate to ASD symptom onset is not well understood, but may relate to the hormones and metabolites produced by the bacteria. Certain species of *Clostridia* that can produce neurotoxins have been found to be specific to individuals with ASD and were not present in control subjects (Finegold et al., 2002).

The Role of the GI microbiota in Neurodevelopment

Brain development extends into postnatal life, providing a window of vulnerability to external insults that could have lasting effects on cognition and behavior (Andersen, 2003). Additionally, many metabolic functions of the developing microbiota including metabolism of vitamins, amino acids and extracting energy form diet, are crucial for brain development (Selkrig et al., 2014). Because, the development of the GI microbiota occurs primarily during infancy parallel to brain development, the GI microbiota could play a principal role in neurodevelopment (Borre et al., 2014). Indeed, studies using GF mice showed a critical phase in the prenatal period in which the GI microbiota significantly influences neurodevelopment (Neufeld et al., 2011; Diaz Heijtz et al., 2011) and is crucial for the normal development of motor activity, anxiety behavior and social behavior (Diaz Heijtz et al., 2011; Desbonnet et al., 2014). Likewise, neurochemical abnormalities (e.g., GABA, BDNF) were observed in GF mice, suggesting that the microbiota plays a central role in brain development (Dinan et al., 2015). Additionally, a "normal" GI microbiota is important for normal development of the hypothalamic-pituitary-adrenal stress system in mice and for myelination in prefrontal cortex (Sudo et al., 2004; Hoban et al., 2016). GF nice show increased levels of synaptic-related proteins as well as myelination proteins, suggesting that the GI microbiota could also be important for normal structural development of the brain (Heijtz, 2016). Accelerated brain growth has been associated with developmental delays in motor, language and cognitive function (Heijtz, 2016). Interestingly, some of the observed neurochemical and behavioral deficits can be reversed during a specific time after birth, suggesting that there is a neurodevelopmental window during which the GI microbiota has a profound effect on behavior and development (Buffington et al., 2016). However, the behavior was normalized only rodents undergoing early colonization and not in

animals undergoing recolonization after many weeks (Neufeld et al., 2011). In a similar study, reconstitution of microbiome in GF mice at 4 weeks but not at 8 weeks reversed social deficit (Buffington et al., 2016). Early life events that can alter the composition of the microbial community (e.g. antibiotic use, hospitalization, breastfeeding or route of birth, Caesarean section) are risk factors associated with higher incidences for developing ASD, suggesting that early life disturbances in the GI microbiota could contribute to the development of ASD (Niehus & Lord, 2006; Curran et al., 2015; Schultz, et al., 2006).

Proposed Mechanisms for the Microbiota-Gut-Brain Communication in ASD

Emerging evidence suggests that the GI microbiota plays a critical role in ASD, but research on the routes of the communication between the gut and brain are limited. In general, proposed mechanisms of the microbiota-gut-brain axis include neural, hormonal, immune and metabolic pathways and a number of systems might be involved simultaneously. For example, interaction between GI bacteria, bacterial metabolites, oxidative stress, dietary allergens, gut permeability and ASD symptoms was described in a connectivity model investigating mechanisms of the microbiota-gut-brain axis in ASD. Several possible pathways of the microbiota–brain interaction in ASD can be proposed as shown in **Figure 1.2**.

GI abnormalities and dysfunction are frequently reported in children with ASD. Intestinal permeability was described in multiple studies with ASD patients which could be caused by changes in composition and metabolic activity of intestinal bacteria, genetic predisposition or dietary components (Bolte, 1998; de Magistris et al., 2010). The reduced integrity of intestinal barrier in turn could lead to increased absorption of toxins from the gut lumen, microbial metabolites or microbes themselves which can impact other organs in the periphery.

The microbiota itself can also have direct effects on the brain by modulating the blood–brain barrier (Collado et al., 2012). Many gram-negative bacteria contain lipopolysaccharides (LPS) on their cell wall which could potentially modulate peripheral tissues including the brain causing an increase in blood brain barrier permeability (Minami et al., 2007) and changes in the metabolism of neurotransmitters such as 5-HT in the brainstem (e.g. *Bifidobacterium infantis*) (Desbonnet et al., 2008). Similarly, animal models have demonstrated that the microbiota provides the brain with other neurotransmitters, such as GABA, acetylcholine and norepinephrine, and influences growth factors such as BDNF (Dinan et al., 2015). Food that escapes digestion by the host can be used by the GI microbiota as energy sources. The GI microbiota is able to release biologically active metabolites and precursors which can be transported by the blood or lymphatic system and act at distal sites such as the brain. This symbiosis marks one of the key mechanisms in the microbiome-brain communication. Bacterial metabolites with relevance to ASD symptomatology include the neurotransmitter serotonin (5-HT) and VFAs.

5-HT has been linked to processes such as social interaction and aggression (Nankova t al., 2014). Genetic, GI or immune changes have been proposed as potential contributing factors to the hyperserotonemia observed in children with ASD (Coutinho et al., 2007; Mulder et al., 2010; Burgess et al., 2006); however, not all cases of hyperserotonemia in children with ASD can be explained by genetic variations. Clinical and preclinical studies have shown that the GI microbiota can influence peripheral and central tryptophan and 5-HT levels, which have the potential to regulate mood and cognition (Jenkins et al., 2016). 5-HT is produced by certain *Lactobacillus*, *Streptococcus*, and *Lactococcus* species (Özoğul et al., 2012) which have been found to be altered in children with ASD. Likewise, sporeforming bacteria can promote 5-HT

synthesis in enterochromaffin cells and modulate concentrations in the colon and blood (Yano et al., 2015). Increased 5-HT production by some strains of the GI microbiota in ASD could deplete peripheral tryptophan availability, which corresponds with data showing decreased 5-HT synthesis capacity in children with ASD as well as reports showing a worsening in repetitive behaviors in individuals with ASD after tryptophan depletion (Chugani et al., 1999; McDougle et al., 1996). In addition, 5-HT cannot cross the blood-brain barrier and, thus, must be produced in serotonergic neurons from tryptophan. Similarly, due to the increased intestinal permeability more 5-HT produced in the GI tract could translocate into the systemic circulation and lead to the elevated levels of blood 5-HT (Gabriele et al., 2014; Ibrahim et al., 2009; de Magistris et al., 2010; d'Eufemia et al., 1996). Lastly, higher levels of 5-HT in children with ASD can be linked intestinal inflammation often observed in children with ASD (Marler et al., 2016). 5-HT has been described to play an important role in intestinal inflammatory responses (Bischoff et al., 2009). Although the cause and effect relationship between dysbiosis and intestinal inflammation is not yet fully understood, intestinal inflammation is associated with increased whole blood 5-HT levels and alterations in the GI microbiome (Butto & Haller, 2016). Thus, it can be proposed that intestinal inflammatory response in children with ASD which is exacerbated by the GI microbiota can lead to a further increase in 5-HT levels and ultimately to upstream behavioral effects on the brain. Interestingly, administration of *Bifidobacteria fragilis* normalized plasma levels of 5-HT in an animal model of ASD (Yano et al., 2015) Likewise, ingestion of *Bifidobacteria infantis* by conventional rats resulted in changes of 5-HT metabolism in the brain stem and increased total plasma tryptophan levels (Neufeld et al., 2011; Desbonnet et al., 2008). These data indicate that the GI microbiota could be involved in higher 5-HT production, thus

supporting the role of 5-HT as a potential pathway whereby the GI microbiota and brain communicate in ASD.

Although to date little is known about the effect of microbiota on hormones of the gut-brain communication, it seems plausible that the GI microbiota can modulate hormonal signaling in the gut-brain-axis. Gut hormone and neuropeptide release from enteroendocrine cells can be regulated by the GI microbiota and gut hormones were proposed to influence cognitive processes (Schéle et al., 2013; Finger et al., 2010; Giordano et al., 2006; Holzer et al., 2012). For example, the release of peptide YY, which shows anxiolytic effects in rats, from intestinal L-cells is stimulated by SCFAs (Holzer et al., 2012, Berglund et al., 2003). Other bacterial metabolites could be involved in the pathophysiology of ASD. In an animal model of ASD, specific bacterial metabolites (4-ethylphenylsulphate; 4EPS) caused ASD-related behaviors, proposing that bacterial metabolites could be a way of communication between the GI microbiota and brain in ASD (Hsiao et al., 2013; De Angelis et al., 2013; Yap et al., 2010; Ming et al., 2012).

Besides acting directly on the CNS, bacterial metabolites may contribute to ASD pathophysiology through alterations of genes associated with ASD mutations or genetic pathways. However, epigenetics mechanisms of the gut-brain-axis have not been extensively studies. It has been hypothesized that microbial products can affect chromatin plasticity, mediate gene-environment interactions or act as an epigenetic entity on its own (Stilling et al., 2014). Evidence for these hypotheses come from studies showing that the GI bacteria can influence host gene transcription by secreting proteins that can act as transcriptional regulators (Bierne, 2013) as well as studies describing the importance of the GI microbiota in neurodevelopment, development of the immune system as well as associations between microbial dysbiosis and disease, including ASD.

Another potential mechanism linking the GI microbiome to behavior in ASD is via VFAs, most notably propionate, which can come from microbial carbohydrate fermentation or be part of food as food preservatives. Due to the ability to cross the blood brain barrier and modulate neurotransmission and behavior, VFAs have been proposed to be neurotoxic (De Vadder et al., 2014; El-Ansary et al., 2012). Elevated levels of propionate in the brain was correlated with decreased concentrations of linoleic, linolenic, arachidonic acid (ARA) and docosahexaenoic acid (DHA) (Thomas et al., 2010). Altered brain phospholipids and acylcarnitine profiles, glutathione depletion, neuroinflammation and oxidative stress were observed in rodents infused intracranially with propionate (El-Ansary et al., 2014; MacFabe et al., 2007). Individuals with impaired propionate metabolism also displayed neurodevelopmental abnormalities similar to symptoms of ASD (MacFabe, 2012, MacFabe, 2015). The precise mechanisms of how VFA alter behavior in ASD are unknown, but effects on mitochondrial function (e.g. Kreb's cycle) or epigenetic alterations may be involved (Reigstad et al., 2015). In addition to direct effects on the brain, propionate has been shown to modulate 5-HT secretion in the gut and deplete 5-HT and dopamine levels in the brain, which could potentially contribute to the hyperserotonemia observed in children with ASD (El-Ansary & Al-Ayadhi, 2014; Reigstad et al., 2015, Al-Ghamdi et al., 2014). Likewise, VFAs can influence catecholaminergic, serotonergic and cholinergic signaling pathways potentially though promoting the release of calcium (Severson et al., 2003). VFAs can also alter host gene expression that have been implicated in ASD pathophysiology such as Fragile X Mental Retardation gene, neurexin 1 or alter genes of carnitine metabolism (Nankova et al., 2014). Interestingly, microbial genera that occur more frequently in children with ASD include propionate producers, such as Clostridia, Bacteroidetes, and *Desulfovibrio* species (Finegold et al., 2010; Parracho et al., 2005). In

addition, in a systems-level model increased levels of *Bacteroides vulgatus* were associated with increased propionate concentrations in the brain in animal models (Downs et al., 2014).

The HPA-axis, a major integrative system for stress adaptation, was found to be another key pathway in the microbiome-brain communication. In a landmark study, Sudo et al. identified the crucial role of microbiota in the normal development of the HPA axis during the early postnatal period (Sudo, et al., 2004). Since then, several studies have shown the bidirectional nature of the HPA-axis (Sudo, 2012; O'Mahony et al., 2011; Gareau et al., 2007). Early life stressors that cause a disruption in the HPA axis regulation also produce long-term changes in the GI microbiota and a healthy microbiome is necessary to develop normal HPA-axis activity (de Weerth, 2017). Other preclinical studies demonstrated an exaggerated HPA axis response in GF mice (Luczynski et al., 2016). Pro- and prebiotic intervention studies in human subjects resulted in a decreased urinary cortisol, the end product of HPA axis activation, decreased cortisol stress reactivity and reduced cortisol awakening response (Schmidt et al., 2015; Allen et al., 2016; Messaoudi et al., 2011). Whether the GI microbiota is involved in regulating the HPA axis activity in the ASD population remains to be determined, but increased activity of the HPA axis in this population has been observed (Spratt et al., 2012). Likewise, the end-product of HPA axis activation, cortisol, was significantly higher in a subgroup of children with ASD compared to controls (Tordjman et al., 2014).

The vagus nerve is another potential avenue of communication between the GI microbiota and the brain. Neural signals from the GI tract to the brain can be transmitted via the vagus nerve, dorsal ganglia root or somatosensory afferents (O'Mahony et al., 2011) Evidence for vagal mediated signaling from the GI microbiota to the brain comes from studies in which behavioral and neurochemical changes induced by some bacterial strains (e.g. *Lactobacillus*

rhamnosus, *Bifidobacterium longum*) were abolished in vagotomized animals (Bravo et al., 2011; Bercik et al., 2011). Two potential mechanisms for the effect of neuroactive bacteria on behavior and neurochemistry were proposed. First, vagal chemoreceptors that are innervating mucosal villi could be activated by bacterial metabolites that can be transported across the epithelial barrier such as SCFA or 5-HT (Forsythe et al., 2015). Second, vagal mechanoreceptors (i.e. intramuscular arrays and intraganglionic laminar endings) could sense intestinal motility changes induced by some beneficial bacteria (Forsythe et al., 2015).

Lastly, the immune system has been implicated as a mediator of the communication between the GI microbiota and behavior. The microbiota could affect microglia development directly and can influence the development of the immune system through various microbial signals (Rea et al., 2016; Kabat et al., 2014; Golnik et al., 2009; Thaiss et al., 2016). For example, pattern recognition receptors can recognize microbial cell components and metabolites and can adjust the immune response accordingly (Thaiss et al., 2016). In addition, disturbance of the gastrointestinal tract can lead to increased permeability of the intestinal barrier, allowing immune cells (e.g. lymphocytes) and cytokines to translocate to the circulation. In the brain, these immune cells can elicit an immune response by increasing the permeability of the BBB or binding to epithelial cells (de Theije et al., 2011). In children with ASD, altered immune function and increased levels of cytokines and other inflammatory proteins has been described (Rodrigues et al., 2014; Ashwood et al., 2010). Thereby, the concentration of cytokines correlated with severity of communication deficits and behavior dysfunction (Ashwood et al., 2010). Neuroimmune pathways have been discussed to contribute to ASD symptomology via the gut-brain axis (de Theije et al., 2011). De Theije et al. proposed that ASD-associated cytokines originating from an inflamed GI tract can cross the blood-brain barrier and elicit an immune

response in the brain, thereby influencing behavior. In a mouse model of psychosocial stress, immunization with the bacterium *Mycobacterium vaccae* promoted better coping in a social stress environment potentially through activation of T-regulatory cells (Reber et al., 2016). Because the microbiome has been shown to be involved in the regulation and development of the immune system, altered GI microbiota associated with GI inflammation might thereby not only contribute to GI disturbances in children with ASD, but also influence behavioral problems through immune cells translocation into the circulation (Collado et al., 2012; Kabat et al., 2014). A positive correlation between expression of interferon signaling-associated genes in the blood and abundance of *Faecalibacterium* in the feces of children with ASD could suggest that abundance of *Faecalibacterium* and *Blautia* might be involved in the dysfunction of systemic immunity that is often observed in children with ASD (Inoue et al., 2016). Endotoxin, a bacterial LPS that can cause neuronal cell death and neuroinflammation, was not only increased in serum of adult patients with ASD but also predicted deficits in the social communication domain of the patients (Emanuele et al., 2010). Through application of a systems level model, it was suggested that the mucosal immune response to bacteria leads to increased systemic inflammatory cytokines which in turn alter the gut and blood brain barrier permeability, allowing translocation of toxins, microbiota and bacterial metabolites into the periphery and the brain (Downs et al., 2014).

Mechanisms underlying the microbiota-brain communication in ASD are heavily investigated. Progress in the understanding of these mechanisms will allow for new opportunities for therapeutic early interventions to ameliorate some symptoms of ASD and potentially prevent other neurodevelopmental deficits and brain disorders. Future research delineating the contribution of each pathway will provide a better understanding microbiome-gut-brain

communication and may identify new therapeutic interventions for neurological diseases such as ASD. Thereby, the use of GF and Gnotobiotic (GN) animal models will be of fundamental importance. The use of GN rodent models has led to significant processes in defining how the GI microbiota regulates body processes, including gut-to-brain communication. However, rodent models are limited by several important physiological and metabolic differences from humans. Therefore, the piglet model is the preferred model for studying human-related host-microbe interactions, due to its similarity to humans in anatomy and physiology (Puiman & Stoll, 2008; Patterson et al., 2008).

Role of Nutrition in the Microbiota-to-Brain Communication

Diet is a major environmental factor regulating the establishment, maturation and maintenance of GI microbiota diversity and abundance (Kang et al., 2014; Thaiss et al., 2016; David et al., 2014; Wu et al., 2011). Indeed, more than 50% of microbial changes can be attributed to diet (Zhang et al., 2010). Although nutrition plays an important role in shaping the GI microbiota and nutrition interventions are commonly used to treat some symptoms of ASD, studies published lack in systematically investigating the effect of dietary habits of children with ASD on the GI microbiota composition (**Table 1.2**). Only Son et al. collected dietary information and analyzed macronutrient content of the diet (Son et al., 2015). However, short- as well as long-term dietary changes can alter the microbial community substantially and children with ASD are often picky eaters, which can have a fundamental effect on the GI microbiota (Wu et al., 2011; Finegold et al., 2002; Martins et al., 2008); thus, it is important to understand how diet modulates the microbiota in the ASD population.

An interrelationship between dietary intake, neurodevelopment, and cognitive function has been demonstrated in healthy children and most results are attributed to the direct effect of dietary components on the central nervous system (Khan etl al., 2015; Khan et al., 2015a). Adequate nutrition has been implied as an important aspect in the treatment and etiology of some psychiatric disorders (Sarris et al., 2015). Thereby, the interaction between diet and behavior could be due to altered metabolism of dietary components and changes in metabolic products as well as direct interaction of the GI microbiota with enteric neurons (Hanstock et al., 2004; Furness et al., 1999). When transferring microbiota of mice fed either a high fat or chow diet to, mice receiving microbiota from high fat fed mice showed increased anxiety like behavior compared to mice receiving microbiota from chow-fed mice (Bruce-Keller et al., 2015), suggesting that diet-induced changes in the GI microbiota could lead to neurological and behavioral changes. Similarly, inoculation of GI microbiota from undernourished children to GF mice caused changes in host metabolism and the immune system, giving additional evidence that diet-induced microbiota composition affects host systems and processes (Kau et al., 2015).

The role of microbial changes through dietary modification on cognitive processes is not well described. In malnourished individuals it has been hypothesized that the microbiome is causally related to neurological abnormalities (Goyal et al., 2015). Thereby, cognitive abnormalities in children that are undernourished might relate to immature GI microbiota. This hypothesis is supported by evidence demonstrating that normal GI microbiota is required for healthy brain development, the bi-directional signaling pathway between the gut and the brain through nerves innervating the GI tract, and the observation that abnormal GI microbiota can result in abnormalities of behavior, cognitive function, structural and neurochemical abnormalities (Goyal et al., 2015). A few animal models have shown that diet-induced changes

in GI microbiota could contribute to observed behavioral changes (Li et al., 2009, Pyndt Jørgensen et al., 2014). For example, rodent fed a meat-containing diet had an increase microbial diversity as well as improved working and reference memory (Li et al., 2009). A high fat diet caused an increase in the abundance of Firmicutes and a decrease in the abundance Bacteroidetes in mice that went along with a decrease in memory function (Jørgensen et al., 2014). Other studies confirmed the effect of high calorie diets on brain function with co-occurring changes in the GI microbiota composition (Magnusson et al., 2015). Interestingly, one study found that feeding a high-fat or high-sucrose diet to rodents resulted in higher percentages of Clostridiales and Bacteroidales, two bacteria orders that have been found to be altered in children with ASD, compared to control mice. Behaviorally, these mice also displayed poorer cognitive flexibility compare to mice fed a normal chow diet (Mangusson et al., 2015). A Western-style diet, which is characterized by high fat intake, negatively affected anxiety-like behavior and memory capabilities and was associated with an increased ratio of Firmicutes to Bacteroides as well as an increase in *Proteobacteria* and *Spirochaetes* (Ohland et al., 2013). Lastly, vitamin and mineral supplementation which was successful in alleviating some symptoms of ASD could also affect the microbiota composition. For example, rodents treated with a magnesium deficient diet had a decrease in bacterial diversity as well as altered anxiety-like behavior (Jørgensen et l., 2015).

A limited amount of studies investigating the potential of dietary interventions to impact cognitive function through the microbiota is available. Associations studies demonstrated that intake of highly processed foods can decrease microbial diversity and was linked to increased risk for mental disorder (Dawson et al., 2016). Pro- and prebiotic supplementation in human subjects are prominent ways to manipulate the microbiota and investigating the effect on brain function. Supplementation with galactooligosaccharide stimulated the growth of *Bifidobacterium*

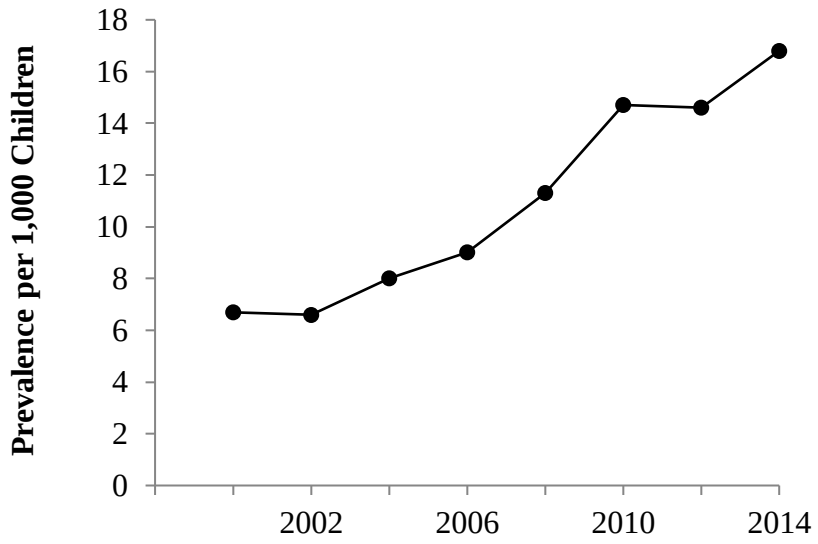
and decreased waking cortisol response (Messaoudi et al., 2011) and probiotic supplementation with *Lactobacillus rhamnosus* GG in early childhood was protective against development of ADHD and Asperger Syndrome at 13 years of age (Pärtty et al., 2015). This study suggests that manipulation of GI microbiota early in life could protect against development of neurodevelopmental disease

In ASD, the GI microbiota could provide a potential link between diet and ASD symptomology. First, dietary interventions and probiotic supplementation alleviate some ASD symptoms and normalized some systemic bacterial metabolites in an animal model of ASD. Second, VFA that are altered in children with ASD and can elicit ASD-like behavior in animal models are a major product of bacterial carbohydrate fermentation. Fiber (e.g., inulin, pectin) present in fruits and vegetables, which is usually low in the diet of children with ASD, decreased propionate production in vitro (Yang et al., 2013). Thus, the substrate available could drive microbial activity in ASD. Lastly, the GI microbiota is capable of producing 5-HT from the amino acid tryptophan and regulating the availability of dietary tryptophan.

Including diet history and detailed diet diaries to clarify relationship between diet patterns and GI microbiota is important to investigate the correlation between nutrient intake and changes in microbiome. Because the diet of children with ASD is often characterized by a lack in variety, an inadequate amount of fiber containing foods and increased amount of sugar containing foods, investigating a relationship between diet and microbial dysbiosis could lead to new potential pre- or probiotic interventions to alleviate symptoms. Future well-designed clinical trial on dietary interventions could be very fruitful in delineating the potential of diet-induced changes in alleviating some symptoms of ASD.

2.7 Tables and Figures

Figure 2.1 Prevalence of ASD (2000-2014)



Adapted from CDC.

Table 2.1 Studies investigating the effectiveness of probiotic supplementation on ASD symptomology.

Citation	Cohort Population	Probiotic Strain studied	Dose used	Key Findings	Dietary Information	Limitations
Human Studies						
Sandler et al. (2000)	11 ASD subjects (10 boys, 1 girl, age 3.5 to 7 years; regressive-onset autism	Vancomycin *Lactobacillus acidophilus, L. bulgaricus, Bifidobacterium bifidum*	Vancomycin: 500 mg/day for 8 weeks; followed by probiotics: $40x10^9$ CFU/mL for 4 weeks	Short term improvement in communication and behavior; No long term improvement after discontinuation of vancomycin treatment; Absent *Peptostreptococcus* and other Anaerobic cocci in feces of children with ASD.	No diet information collected.	Poor compliance to probiotic treatment. No control subjects (microbiome was compared to adult microbiome)
Kałużna-Czaplińska et al. (2012)	22 ASD subjects; 4-10 years of age; 2 female, 20 male; severe GI symptoms	*Lactobacillus acidophilus* (strain Rosell-11)	$5x10^9$ CFU/g; 2 times/ day for 2 months	Probiotic supplementation decreased D-arabinitol concentration and ratio of D-/L-arabinitol in urine	ASD subjects were on sugar free diet and consumed either a varied or restricted diet. Other dietary supplements were used (magnesium, vitamin B2, B6, Cod liver oil)	Microbiome composition was not analyzed, No control subjects

Table 2.1 (cont.)

Citation	Cohort Population	Probiotic Strain studied	Dose used	Key Findings	Dietary Information	Limitations
West et al. (2013)	33 ASD subjects; 3-16 years of age	Delpro® (*Lactobacillus acidophilus, L. casei, L. delbruecki, Bifidobacterium longum, B. bifidum*); Formulated with immunomodulator Del-Immune V® (*Lactobacillus rhamnosus* V lysate)	$1x10^{10}$ CFU; 3 times/ day for 6 months	Decreased severity of ASD symptoms; Improvement of GI symptoms	Lack of information on diet	Microbiota composition was not analyzed; No control subjects
Tomova et al. (2015)	10 ASD subjects (2-9 years, 9 boys, 1 girl) vs. 9 non-ASD siblings (5 to 17 years, 7 boys, 2 girls) vs. 10 non-	3 strains of Lactobacillus (60%) 2 strains of Bifidobacterium (25%) 1 strain of Streptococcus (15%)	3 times/ day for 4 months	GI dysfunction was higher in ASD children and siblings compared to controls at baseline; Strong positive correlation between GI symptoms and ASD severity; Increased Bacteroidetes/Firmicutes ratio after probiotic supplement; Decreased Firmicutes abundance after probiotic treatment; Decreased in	Lack of information on dietary habits	Lack of information on exact dose of probiotic strains; Only ASD group underwent probiotic treatment; Changes in ASD

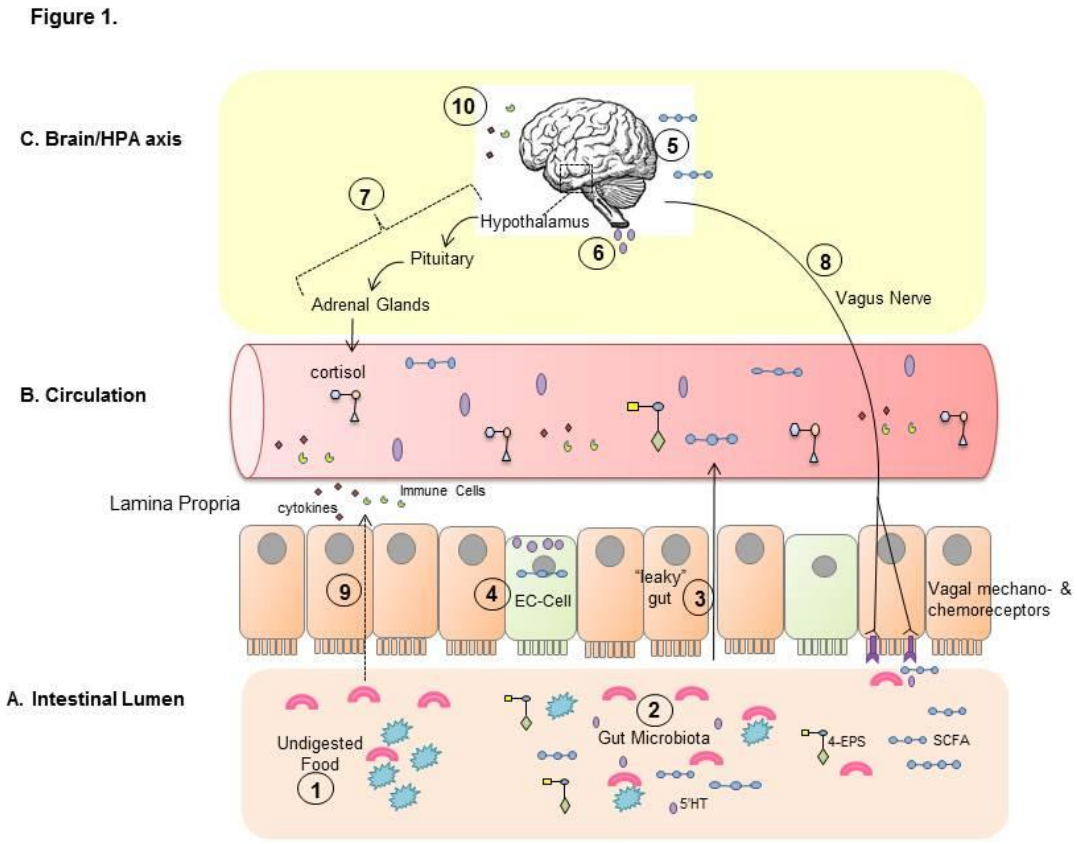
Table 2.1 (cont.)

Citation	Cohort Population	Probiotic Strain studied	Dose used	Key Findings	Dietary Information	Limitations
	ASD controls (2 to 11 years, 10 boys)			*Bifidobacterium* and *Desulfovibrio* after probiotic treatment; Children with more severe ASD had higher Clostridia and *Desulfovibrio* and lower Bacteroidetes/Firmicutes ratio; Correlation between TNFα levels and GI symptoms and trend toward correlation of TNFα and ASD severity; Probiotic supplementation decreased TNF α levels		symptoms after probiotic treatment were not reported.
Russo et al. (2015)	49 ASD subjects: (39 males, 10 females) mean age 11.4 years, 26 of these were taking probiotic therapy, subjects were diagnosed with GI	No information provided	No information provided	Children with ASD taking probiotic therapy had decreased plasma levels of myeloperoxidase (MPO), decreased plasma copper but not zinc levels.	Dietary information was not collected	Microbiome was not analyzed; No information on which probiotic was used by the subjects;

Table 2.1 (cont.)

Citation	Cohort Population	Probiotic Strain studied	Dose used	Key Findings	Dietary Information	Limitations
	disease) vs. 36 control subjects (29 males, 7 females) mean age 10.2 years					Controls were not assessed for GI disease
Animal Studies						
Hsiao et al. (2013)	maternal immune activation (MIA) mice model of ASD	*Bacteroides fragilis* (ATCC 9343)	$1x10^{10}$ CFU; every other day for 6 days at weaning	corrected GI barrier integrity, alterations in tight junctions and cytokine expression; corrected relative abundance of specific groups of microbes in Lachnospiraceae family and unclassified Bacteriodales; amelioration in MIA-associated dysbiosis; amelioration in ASD-like behaviors (improvements in communicative, repetitive, anxiety-like and sensorimotor behavior but not sociability and social preference)		

Figure 2.2 Nutrition and the Microbiome-Gut-Brain Axis in ASD.



From: Berding et al. Nutr Rev. 2017

Possible pathways of the microbiome-brain interaction in ASD can be proposed. Food that escapes digestion by the host can be used by the GI microbiota as energy sources. In turn, bacterial metabolites (e.g. SCFAs, 5-HT) are produced that can be used for physiological functions by the host. (**1**); Bacterial metabolites with relevance to ASD symptomology include 5'HT and SCFA. 5'HT is produced by certain Lactobacillus, Streptococcus and Lactococcus strains (Özoğul et al., 2012). Microbial genera that are greater in children with ASD include propionate producers, such as Clostridia, Bacteroidetes and Desulfovibrio (Finegold et al., 2010; Parracho et al., 2005). Increased 5'HT production by the microbiota could lead to tryptophan depletion and contribute to hyperserotonemia observed in ASD. (**2**); Bacterial metabolites can be translocated into systemic or lymphatic system, transported to the brain and cause behavioral and chemical changes. In addition, abnormal intestinal permeability in children with ASD could

Figure 2.2 (cont.)

allow passive diffusion of metabolites (de Magistris et al., 2010) (**3**); Although 5'HT is predominantly produced from EC-cells in enterocytes, its secretion can be stimulated by SCFAs which could increase the amount 5'HT is released into the circulation (Reigstad et al., 2015) (**4**); When reaching the brain SCFAs can have neurotoxic effects and propionate could elicit ASD like behavior in animal models (de Vadder et al., 2014; MacFabe, 2012) (**5**); The microbiome itself can have direct effects on the brain by modulating the BBB and causing changes in the metabolism of 5'HT in the brainstem (e.g. *Bifidobacterium infantis*) (Collado et al., 2012; Desbonnet et al., 2008). (**6**); Certain strains of the GI microbiota have been shown to influence the activity of the HPA axis (Sudo et al., 2012; Sudo, 2004; Gareau et al., 2007). In children with ASD, increased activity of HPA axis as well as increased levels cortisol in the circulation were observed (Tordjman et al., 2014). (**7**); The vagal mediated signaling from the GI microbiota to the brain can be transmitted through vagal chemoreceptors on mucosal villi that are activated by bacterial metabolites (e.g. 5'HT, SCFA) or by vagal mechanoreceptors which sense motility changes induced by some bacterial species (Forsythe et al., 2015) (**8**); The microbiome can influence the development of the immune system through various microbial signals (Kabat et al., 2014; Golnik & Ireland, 2009; Thaiss et al., 2016). For example, pattern recognition receptors (PPRs) can recognize microbial cell components and metabolites and adjust the immune response accordingly (Thaiss et al., 2016). In addition, disturbance of the GI tract can lead to increased permeability of the intestinal barrier allowing immune cells (e.g. lymphocytes) and cytokines to translocate in the circulation (**9**); In the brain, these immune cells can elicit immune response by increasing permeability of BBB or binding to epithelial cells (de Theije et al., 2011). (**10**)

Abbreviations: 5'HT: serotonin, SCFA: short chain fatty acids; EC-cells: enterochromaffin cells; GI: gastrointestinal; BBB: blood brain barrier

Table 2.2 Dietary information provided in human studies investigating microbiota composition in ASD children.

Publication	Dietary Information Provided
Horvath et al. (1999)	GF/CF diet
Finegold et al. (2002)	"Many of the patients were on a GF/CF diet"
Parracho et al. (2005)	Varied or restricted (GF/CF) diet
Wang et al. (2011)	GF/CF diet, probiotics, antibiotics
Adams et al. (2011)	probiotic use, seafood and fish oil consumption
Kang et al. (2013)	"information on special diets (e.g. GF/CF, nutritional supplements, probiotics, seafood)"
Son et al. (2015)	Dietary information collected for 7 days prior to collection of stool samples; analyzed total kcal, protein, fat carbohydrate, fat, fiber, sugar

Abbreviations: GF/CF: gluten free, casein free

CHAPTER 3

Fecal Microbiome Composition and Stability in 4- to 8-Year Old Children is Associated with Dietary Patterns and Nutrient Intake[2]

Abstract

Background/Aims: Previous studies have reported that dietary intake can shape the microbiota composition and stability in adults. How long-term dietary intake shapes microbiota composition and stability in young children is poorly understood.

Methods: Herein, the temporal variability in stool microbiota composition in relation to habitual dietary patterns of 4 to 8 year-old children (n=22) was investigated. Fecal samples were collected at baseline, 6 weeks and 6 months. Bacterial composition and volatile fatty acids were assessed by 16S rRNA sequencing and gas-chromatography, respectively. Nutrient intake was assessed using 3-day food diaries and dietary patterns were empirically derived from a food frequency questionnaire.

Results: Using a factor loading of >0.45 for a food group to be a major contributor to the overall dietary pattern, two dietary patterns were found to be associated with distinct microbiome composition. Dietary Pattern 1 (DP1), characterized by intake of fish, protein foods, refined carbohydrates, vegetables, fruit, juice and sweetened beverages, kid's meals and snacks and sweets, was associated with higher relative abundance of Bacteroidetes, *Bacteroides* and *Ruminococcus* and lower abundance of *Bifidobacterium*, *Prevotella*, *Blautia* and *Roseburia*. Dietary Pattern 2 (DP2), characterized by intake of grains, dairy and legumes, nuts and seeds, was associated with higher relative abundance of Cyanobacteria and *Phascolarctobacterium* and

[2] This article appeared in its entirety as Berding K, Holscher HH, Arthur AE, Donovan SM. Association of dietary patterns with composition and stability of the gut microbiota in children. *J Nutr Biochem*. 2017. 20:56:165-174. This article is reprinted with the permission of the publisher.

lower abundance of *Dorea* and *Eubacterium*. Fruit and starchy foods were present in both patterns, but were more associated with DP1 and DP2, respectively. Temporal stability of microbiota over a 6-month period was associated with baseline dietary patterns.

Conclusion: Understanding how dietary intake contributes to microbiota composition and stability in early life in important for dietary recommendations and designing clinical interventions for microbiota-associated diseases.

3.1 Introduction

The human gastrointestinal (GI) microbiota is a complex community containing an estimated 500 species and approximately 3 million bacterial genes, which benefit the host by providing protection against pathogens and programming the immune response (Weinstock, 2012; Kabat et al., 2014; Servin, 2004). Recent research has revealed that the GI microbiota is also involved in brain development and cognitive processes and microbial dysbiosis has been associated with various cognitive disorders, including Autism Spectrum Disorder (Son et al., 2015; Kang et al., 2013; Andersen, 2003; Diaz Heijtz et al., 2011; Berding & Donovan, 2017). Diet is considered one of the most influential environmental factors in determining the composition of the gut microbiota. It has been hypothesized that due to shifts in dietary habits in the modern culture, diet-induced microbial changes are involved in diseases such as inflammatory bowel disease, allergies or other autoimmune diseases (De Filippo et al., 2010). It has been suggested that longer-term dietary patterns can change the microbiota over a longer period of time that short-term changes in dietary habits cannot accomplish (David et al., 2014). Dramatic short-term changes over 5-days in intakes of dietary fat, protein, and fiber has been shown to impact the composition and function of the human GI microbiota, while habitual

dietary patterns are thought to be more notably associated with long-term stability of the microbiota (Lozupone et al., 2012; Wu et al., 2011). In one study, a short-term (10 days) dietary intervention did not lead to a change the bacterial enterotype that was associated with an individual's long-term dietary patterns (Wu et al., 2011). Thus, the interest in investigating the impact of dietary patterns on the composition of the microbiota has led to crucial advancements in the understanding of how habitual eating patterns affects the bacterial profile.

Studies in humans and other mammals have shown that the gut microbiota composition can be clustered based on diet (herbivore, omnivore or carnivore) (Muegge et al., 2011). Additionally, individuals following a vegetarian diet have higher phylogenetic diversity compared to diet patterns rich in meat or mixed diets (Ley et al., 2008). Greater diversity is associated with greater potential for resistance to change, which suggests that a plant-based diet may be more beneficial in maintaining stable GI microbiota throughout environmental challenges. The abundance of *Bacteroides* has been associated with higher intakes of animal protein, amino acids and saturated fatty acids, whereas the abundance of *Prevotella* was associated with high intakes of carbohydrates, fibers and simple sugars (Wu et al., 2011). Dietary patterns, such as the Western Dietary Pattern which is characterized by high-fat/high-sugar foods, have been associated with poorer health outcomes and increased risks for developing metabolic disease, including obesity, type 2 diabetes, and cardiovascular disease, compared to dietary patterns that are higher in whole foods, fruits and vegetables (Albenberg & Wu, 2014). Diet-induced changes in microbiota composition can lead to increased risk of developing certain diseases (e.g., inflammatory bowel diseases), whereas a healthier long-term dietary pattern may be more beneficial in promoting a microbial profile that could potentially protect against diseases (Albenberg & Wu, 2014). For example, dietary patterns of obese individuals were associated

with microbial gene diversity and quantity which might impact host metabolic and inflammatory markers (Koenig et al., 2011).

The microbiota undergoes rapid changes in the first years of life (Bergström et al., 2014). Although previous studies have suggested that the gut microbiota becomes relatively stable and resembles that of an adult at 3 years of age, other studies suggest that the gut microbiota might have a more prolonged development, lasting into pre-adolescence (Palmer et al., 2007; Yatsunenko et al., 2012; Hollister et al., 2015; Agans et al., 2011). Given that the GI microbiota can potentially contribute to the development of diseases in genetically at-risk individuals, it is important to understand whether specific long-term dietary patterns are associated with a more beneficial microbiota that could show a higher resiliency to environmental challenges. Therefore, the goal of this longitudinal observational study was to investigate the GI microbial composition of children aged 4-8 years and determine whether associations exist between microbial composition and specific dietary patterns. We hypothesized that children with an eating pattern containing fiber-rich foods such as fruits, vegetables, legumes, and whole grains, would be associated with a more diverse microbiota which would be more stable over time, whereas a diet high in animal products and processed foods would be associated with less beneficial bacterial composition (e.g., butyrate producers, bacterial species known to protect against pathogens) and would be more variable over time.

3.2 Materials and Methods

Participants and Baseline Questionnaire

Children between 4 and 8 years-of-age (n=22) were recruited in the Champaign-Urbana, IL area between April 2016 and September 2016. Subjects were recruited as part of a larger

study currently being conducted in the laboratory. Data presented herein was selected to serve as a pilot analysis to investigate the impact dietary patterns on the GI microbiota in children. All subjects were free of functional digestive disorders, had not used antibiotics, probiotics or prebiotic in the 3 months prior to enrollment in the study, did not take any medication and did not follow any special diet (e.g., gluten-free/casein-free diet). Parents completed a baseline questionnaire including questions regarding their child's age, gender, early feeding practices, mode of delivery, nutritional supplement use, height and weight. The height, weight and BMI of the participants were converted to percentiles according to the CDC growth charts for both male and female participants. Questionnaires at the 6-week and 6-month time points included questions on newly introduced medications and changes in gastrointestinal health in order to control for potential impact on changes in GI microbiota. Participants provided oral assent and their legal guardians provided written consent in accordance with the ethical standards of the Institutional Review Board of the University of Illinois at Urbana-Champaign.

Fecal Sample Collection, DNA Extraction and 16S rRNA Sequencing and Analysis

For each subject, freshly-voided morning stool samples were collected at enrollment (baseline), 6-weeks and 6-months post-baseline for the analysis of the fecal microbiota and volatile fatty acids (VFA) concentrations. Stool samples were collected into a plastic commode specimen collection system (Fisher Scientific, Waltham, MA). Parents were provided with gloves and a sterile spoon to transfer ~5 to 10 g of freshly-voided fecal material into a sterile 50 mL conical tube. All samples were immediately placed in the participant's freezer (-20°C) until transported to the laboratory within 30 min of sample collection. All samples were stored in the laboratory at -80°C prior to analysis.

Microbial DNA was extracted from stool using a bead beating method followed by a combination of QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) and the FastPrep-24 System (MP Biomedicals, Carlsbad, CA), as previously described (Li et al., 2012). DNA concentration was measured using a NanoDrop 1000 spectrophotometer. Amplification of the V3 to V4 regions (ca. 430 bp) of 16S rRNA gene was performed using dual-index paired-end sequencing approach using primers F357 and R805 (Klindwirth et al., 2013). The AccuPrime™ Taq DNA Polymerase System (Life Technologies, Grand Island, NY) was used for PCR amplification in a DNAEngine (Bio-Rad, Hercules, CA). The amplicons were mixed in equimolar concentration and sequenced at the W. M. Keck Center at the University of Illinois, Urbana-Champaign. Paired-end sequencing (2 x 300 bp) was performed with an Illumina MiSeq (Illumina, Inc., San Diego, CA) using version 3 chemistry.

The 16S rRNA sequences were processed and analyzed using the QIIME 1.9.1 bioinformatics package (Caporaso et al., 2010). Briefly, sequences were demultiplexed and clustered into operational taxonomic units (OTUs) using the closed-reference OTU picking algorithm with default parameters against the Greengenes 13_8 reference OTU database at a 97% similarity level. Singletons and OTUs with an abundance lower than 0.005% were removed (Bokulich et al., 2013) prior to rarefying the OTU table to a sampling depth of 39,140 sequences per sample using the alpha_rarefaction command. The rarefied PTU table was used for subsequent analysis.

VFA Analysis

Sample preparation for VFA analyses was performed as follows: fecal material (0.1 g) was weighed and mixed with 0.4 mL of 3.25% m-Phosphoric acid solution in a 2 mL

microcentrifuge tube. Samples were vortexed to disperse the sample in solution. After an incubation time of 30 min, samples were frozen over night at -20°C. The next day, the samples were thawed and centrifuged in microfuge tubes at 13,000×g for 10 min. The supernatant was transferred into gas chromatography vials.

The VFA concentrations were analyzed by a Hewlett-Packard 5890A Series gas chromatograph and a glass column (180 cm 3 4 mm i.d.), packed with 10% SP-1200/1% H3PO4 on 80/100 mesh Chromosorb WAW (Superlco). Oven temperature, detector temperature, and injector temperature were set at 1258°C, 1758°C, and 1808°C, respectively. VFA production was calculated as VFA concentrations of substrate-containing tubes minus the VFA content of blank tubes divided by substrate weight expressed on a dry matter basis (Li et al., 2012).

Dietary Patterns

A revised version of the semi-quantitative Youth and Adolescent Food Frequency Questionnaire (YAQ) was completed by the parents to estimate their child's usual food and beverage intake over the past year (Bandini et al., 2010). The questionnaire was modified for parent report as previously described (Bandini et al., 2010). The revised YAQ contained 156 food items, compared with the original 169. The original YAQ has been tested for reproducibility and validity (Rockett et al., 1997). Parents were asked to estimate the frequency with which their children consumed a specified portion size of each of the foods listed over the preceding year. The questionnaire has five possible responses ranging from "never or less than once per month" to "more than 4 times per week". The 156 foods items were grouped *a priori* into 13 food groups (e.g. fruits, vegetables, grains, sweets) (**Appendix B**). using methods similar to those described in previous studies of dietary patterns and disease (Evans et al., 2012). Parents

completed the YAQ at all three sample collection time points. However, for purpose of this analysis only baseline dietary patterns were included. To estimate the number of servings of any food group, each response was converted to the corresponding frequency factor (Harvard T.H Chan School of Public Health Nutrition Department's Food Group Serving Table) and summed over all the food items to get the average servings of a specific food group per day.

3-Food Diary

To monitor short-term nutrient intake prior to fecal sample collection, a 3-day dietary food record was completed at each sample collection time point the 3 days prior to sample collection. The parents were asked to report all types and quantities of food and beverages consumed by their children during the three-day (two week days, one weekend day) period prior to each fecal sample collection. Parents were trained by a member of the research team on how to record their child's meal on the food record. Additionally, parents were provided with a detailed description of how to keep a food record. After collection of the food record a member of the research team followed up with the parent if additional detail was needed. The food diary data were analyzed using the Nutrition Data System for Research (NDSR, Minneapolis, MN, 2014) software.

Assessment of Gastrointestinal Symptoms

Parents completed a questionnaire assessing the severity of their child's GI symptoms, including constipation, diarrhea, stool smell, flatulence and abdominal pain in an adapted version of the Gastrointestinal (GI) Severity Index (Adams et al., 2011a). Scores for each item were

summed to determine an overall severity score. Additionally, stool consistency was assessed using the Bristol Stool Chart (Lewis & Heaton, 1997).

Statistics

Descriptive statistics (means and frequencies) were generated for all demographic and epidemiologic variables of study participants. Dietary patterns data derived from the YAQ were derived using Principal Component Analysis and Factor Analysis with Varimax rotation. Retention of the number of factors in the final analysis was based on Eigenvalues ≥ 2, percentage of variability explained, interpretability of factors and Scree plots. Food groups with a factor loading >0.45 were considered significant contributors to each overall dietary pattern. Factor scores were calculated for each participant by summing the reported frequency of intake for each food in a food group standardized by the factor loadings. Scores for dietary patterns were calculated for each participant and stratified as either falling above or below the median. All subsequent data presented in the manuscript was analyzed for significant differences between the two groups (above vs. below median).

Differences in microbial community structure were evaluated with principal coordinate analysis (PCoA) and permutational multivariate analysis (PERMANOVA) of variance using weighted and unweighted UniFrac distance in the QIIME software. Differences between the groups for each dietary pattern in alpha-diversity (Chao1, Shannon Index and observed OTUs), relative abundance of individual phyla and genera, VFA and nutrient intake data derived from the 3-day food records at the baseline sample collection were analyzed using Wilcoxon Rank Sum Test. Categorical variables were analyzed for significant differences using Chi-Square Test. Longitudinal dynamics in the microbiota composition and changes in dietary intake derived from

the 3-day food record were analyzed for significant differences between the two groups (above vs. below median) using mixed models. Normally distributed data were analyzed using the PROC MIXED procedure and non-normally distributed outcomes were analyzed using PROC GLIMMIX procedure. Each model for the microbiota analysis was controlled for factors known to affect the gut microbiota composition including age, gender, BMI, season of sample collection and dietary fiber intake as well as nutrients whose intake changed significantly over the 6 months study period. Correlations between specific food groups and bacterial abundance were assessed using Spearman correlation coefficient. Data are expressed as mean ± SD. Level of significance was set at $p \leq 0.05$ and $p \leq 0.10$ was considered a trend. All data were analyzed using SAS 9.4 (SAS Institute, Cary, NC).

3.3 Results

Participant Characteristics

A sample of 22 subjects was selected for analysis for this manuscript. All three samples were collected from 20 subjects; however, 2 subjects failed to provide a sample at the 6-months sample collection. Thus, 22 samples were collected for baseline and 6 weeks and 20 samples were collected for the 6-month time point. The study population demographics are shown in **Table 3.1**. The mean age of participant was 5.9 ±1.3 years and most participants were male (63%) than female. The participants self-identified as Caucasian (64%), Asian (14%), Black or African American (14%), and Hispanic or Latino (9%). Parents of participants were well educated with 77% having either a college degree or post-graduate work. Most participants had been exclusively breastfed (45%) for at least 6 months or were breast-fed in combination with formula (50%). Fifty-nine percent were delivered vaginally, 32% were delivered by emergency

cesarean-section, and 9% by planned cesarean-section. The mean BMI for female participants was 17 (85th percentile), whereas the mean BMI for male participants was 16.4 (75th percentile). Half of the parents reported that their child was taking a multivitamin at the time on enrollment into the study.

Dietary Patterns at Baseline

Using factor loading analysis, two distinct dietary patterns were identified at baseline. Dietary Pattern 1 (DP1) was characterized by an intake of fish, refined carbohydrates, vegetables, juice and sweetened beverages, protein foods, kid's meals, condiments and snack and sweets. Dietary Pattern 2 (DP2) was characterized by an intake of grains, dairy and legumes, nuts and seeds. Fruit and starchy foods were present in both patterns, but were more associated with DP1 and DP2, respectively. A factor loading matrix can be found in **Table 3.2**.

Dietary Patterns, Participant Characteristics, Microbiota Composition and VFA Concentration at Baseline

Participant characteristics, nutrient intakes and bacterial abundance dichotomized by category of factor score (above or below the median) for each dietary pattern are shown in **Tables 3.3-3.5**. Factor scores were derived by summing the reported frequency of intake for each food in a food group standardized by the factor loadings for each participant. A median was calculated and scores were stratified as either falling above or below the median for each dietary

pattern. No significant differences in age, gender, BMI, race or nutritional supplement use were across factor score categories for either dietary pattern.

No differences in the alpha- or beta-diversity or VFA concentrations at baseline between factor score categories in either dietary pattern were observed. However, the dietary patterns were associated with differences in GI microbiota profiles. Participants that fell above the median in the DP1 had a significantly higher intake of daily servings of fruits, vegetables, refined carbohydrates, fish and kids' meals, but a lower intake in servings per day of juice and sweetened beverages and grain foods. Participants that fell above the median in DP1 also consumed more cholesterol and trans fatty acids, but less vitamin A and whole grains. The bacterial profile of participants above the median in DP1 was characterized by higher relative abundances of Rikenellaceae, Bacteroidetes, *Bacteroides*, *Lachnospira*, *Ruminococcus*, and *Hafnia*, but lower relative abundances of Clostridiaceae, Erysipelotrichaceae, *Bifidobacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Blautia*, *Roseburia*, and *Campylobacter* compared to children that fell below the median.

Children that fell above the median in the DP2 had lower intakes of servings per day of vegetables, but higher intakes of legumes, nuts and seeds, dairy foods, and snacks and sweets. Nutrient data from the 3-day food diary showed that children above the median in the DP2 also consumed more total saturated fatty acids and calcium, but less vitamin C compared to children that fell below the median. The bacterial profile of children above the median for DP2 was associated with lower relative abundances of Cyanobacteria and higher relative abundances of *Dorea* and *Eubacterium*.

Changes in Dietary Intake and Food Group Intake from Baseline to 6-Month Based on 3-Day Food Diary

Nutrient intake from the 3-day food records were analyzed for changes over the 6-month period to determine the potential impact on the variability of the gut microbiota over time. Children falling above the median in the DP1 tended to have the highest intakes of riboflavin ($p=0.07$), selenium ($p=0.09$), sodium ($p=0.07$) and lowest intake of Kid's meals ($p=0.0008$) at the 6-week time point. Additionally, an increase in consumption of grains ($p=0.003$) was observed. Children falling below the median in DP1 tended to consume more animal protein ($p=0.09$), but less vegetable protein ($p=0.02$), and omega-3 fatty acids ($p=0.07$) at the 6-week time point. Additionally, a decrease in vitamin D intake ($p=0.03$) and an increase in Kid's meals ($p<0.0001$), and fish ($p=0.09$) over the 6-month study period was observed in children falling below the median in DP1.

Overall, there were no significant changes in macro- or micronutrient intake for children falling below the median in the DP2. However, an increase in consumption of snack and sweet ($p=0.05$), vegetables ($p=0.09$), fruit ($p<0.0001$), legumes ($p=0.09$) and juice and sweetened beverages ($p=0.04$) was observed in children falling below the median in DP2. Children that had scores above the median in the DP2 tended to have an increase in total protein intake ($p=0.09$), intake of fruit ($p=0.003$) and fish ($p=0.003$) over the 6-month period and had the highest intake of animal protein ($p=0.02$) at the 6-week time point. Likewise, consumption of vitamin D ($p=0.0002$) and selenium ($p= 0.07$) were also highest at the 6-week time point in children falling above the median in DP2.

Microbiota Stability over 6-Month based on Dietary Pattern

Significant changes in the bacterial phyla and genera over the entire study period based on dietary pattern are shown in **Table 3.6.** There was no statistically significant difference in alpha- or beta-diversity in either dietary pattern over the 6-month period (data not shown). Participants below the median in the DP1 tended to have a decrease in the abundance of Verrucomicrobia from baseline to 6-weeks and 6-months. At the genus level, children below the median in the DP1 tended to have a decrease in the relative abundance of *Paraprevotella*, and *Lachnospira*. Examining longitudinal stability of the gut microbiota based on DP1 showed that children above the median did not show any significant changes in the composition on the phyla level.

Overall, children above the median in DP1 had more changes in the abundance of bacterial phyla and genera compare with children below the median. At the phyla level, changes in the abundance of Actinobacteria and Bacteroidetes were observed, with Actinobacteria showing the lowest and Bacteroidetes having the highest abundance at the 6-week point. Abundance of Firmicutes increased over the 6-months study period in children above the median in DP1. At the genus level, the abundance of *Bifidobacterium* and *Streptococcus* tended to be lowest at the 6-week time point in children above the median in DP1. Likewise, the abundance of *Roseburia* significantly decrease over the 6-months period. The abundance of *Lachnospira* was significantly higher at the 6-week time point compared to baseline and 6-month time point in children below the median. Children below the median showed a significant decrease in the abundance of Cyanobacteria and Verrucomicrobia over the 6-month study period with the 6-week time point showing the lowest relative abundance in this group.

VFA Concentrations at Baseline Based on Dietary Pattern and Longitudinal Changes

VFA concentrations did not differ between above or below the median group in either dietary pattern (**Table 3.5**). Longitudinal changes of VFA concentrations are shown in **Figure 3.1**. Acetate concentration was significantly increased (p=0.05) from baseline to 6 months in children above the median in the DP1. Alternatively, butyrate concentrations significantly increased (p=0.03) in children below the median in the DP1. In DP2 there was no change in the concentration of any VFA in children below the median, but acetate (p=0.01) and butyrate (p=0.02) concentrations significantly increased from baseline to 6 months in the group of children above the median.

There were no changes in the concentrations of propionate or the branch-chain fatty acids (valerate, isovalerate or isobutyrate) in either dietary pattern.

GI Severity Index and Bristol Stool Chart

Children above the median in DP1 tended to have higher GI severity index scores compared to children below the median (p=0.07). In DP2, children below the median had higher (p=0.004) GI severity scores than children above the median. We also performed regression analysis to assess whether specific bacterial genera and phyla that were higher in the respective groups could predict the GI severity score. A trend (p=0.08) for predicting GI severity based on Cyanobacteria and *Phascolarctobacterium* relative abundance was observed for DP2. There were no statistically significant differences in stool consistency based on the Bristol Stool Chart criteria.

Correlations between Food Groups and Microbiota Abundance

Correlations between fiber intake and intake of specific food groups with microbial diversity and abundance were analyzed for the whole cohort using baseline data. Only baseline data were used for this analysis due to the ability to control for factors such as probiotic, antibiotic or medication use. Several associations between fiber intake and intake of specific food groups were found. The strongest correlations are shown in **Figure 3.2**. Servings per day of refined carbohydrates were negatively correlated with Chao 1 Diversity Index (ρ=-0.48; p=0.03). Servings per day of snacks and sweets was negatively correlated the Shannon Diversity Index (ρ=-0.41; p=0.05). Both *Bacteroides* (ρ=0.66; p=0.008) and Bacteroidetes (ρ=0.52; p=0.01) abundance were positively correlated with the servings of fruit per day.

3.4 Discussion

Habitual dietary patterns have been shown to influence the composition and long-term stability of the gut microbiota in adults, but the association between dietary patterns, microbial composition, and stability of the GI microbiota in children is poorly understood. To fill this gap, we used exploratory Principal Component Analysis to investigate dietary patterns derived from a food frequency questionnaire and the impact on microbiota composition. Two distinct microbiota profiles existed based on dietary patterns. Furthermore, a dietary pattern characterized by higher intakes of vegetables, fruit and grains and lower intakes of starchy foods and juice and sweetened beverages was associated with a greater microbiota stability over a 6-month period. These observations correspond to our hypothesis that a dietary pattern containing fiber-rich foods, including fruits, vegetables and grain, will be associated with a more stable microbiota composition. Thus, different long-term eating patterns in children aged 4-8 years result in

distinct microbial profiles and might have different effects on the temporal variability of the microbiota.

Overall, the macronutrient intake of this study population was characterized by higher than recommended intake of calories from fat (~35% of total calories compared to AMDR 25-35%) and from saturated fats (~13% of total calories compared to recommended 7%), but lower than recommended intakes of calories from protein (~16 % of total calories compared to AMDR 10-30%). Likewise, fiber intake averaged at 15 g at all 3 time points, which is ~ 69% of recommended intake. On the other hand, sodium intake was at 130% of the Dietary Reference Intakes. These observations are similar to previous studies showing that children in the U.S. exceed amounts of recommended intakes of total fat, saturated fat and sodium, but consume well below the recommendations for daily fiber intake (Bronner, 1996; McGill & Devareddy, 2015). Children in the U.S. also often fail to meet the dietary guidelines for fruit, vegetable and whole grain intake. In some food groups, including vegetables and whole grains, children in the U.S. are only meeting half the recommended intake per 1000 kcals (Frazier-Wood et al., 2016). Only 10% of U.S. children consume recommended amounts of fruit and vegetables (Krebs-Smith et al., 2010), which is similar to results from this study. Overall, children consumed less than the recommended servings per day of fruit, vegetables, legumes and seeds, and whole grains, but exceeded the recommended 2-3 servings per day for dairy foods. The similarities in dietary intake of children in this study to nationally-representative populations could suggest that our microbiota findings could be generalizable to young children in the U.S. However, our small sample size does not allow for a definite conclusion but could serve as preliminary evidence for future larger scale studies. Interestingly, most children with a Hispanic background fell in the below median group in DP1, which was characterized by low intakes of fruits and vegetables,

but higher intakes of juice and sweetened beverages. Previous reports found that fruit or vegetable intake differed by race and ethnicity (Kirkpatrick et al., 2012) and children with a Hispanic background often did not meet recommendations for fruits and vegetables (Basch et al., 1994).

Understanding the long-term variability of the GI microbiota is important as resistance to environmental stressors and rapid return to a baseline state are key features of a healthy microbiome (Bajaj et al., 2012). Although extreme shifts in dietary macronutrient and fiber intakes are associated with short-term changes in the gut microbiota, most studies have found that these shifts are reversible and that the GI microbiota returns to baseline composition (Lozupone et al., 2011; Li et al., 2017). Variation in the microbiota is associated with non-dietary factors; thus, age, gender, race, BMI and season of sample collection were included as co-variates in our analyses (Hollister et al., 2015; Dominianni et al., 2015). Additionally, nutrients and food groups that were shown to be different between the time points and introduction of any medication or changes in GI health were included as co-variates. Other studies found that environmental factors can affect the abundance of specific microbial genera in an otherwise stable bacterial core in adults (Bäckhed et al., 2012; Rajilić-Stojanović et al., 2013; Martínez et al., 2013) and bacteria that are lower abundance might be more transient and prone to temporal variations than predominant phyla of the bacterial core (Durbán et al., 2012; Vanhoutte et al., 2004; Caporaso et al., 2011). We hypothesized that a dietary pattern characterized by high fiber-foods would be associated with a more diverse and stable microbiota over time. In this study, we did not observe changes in alpha- or beta-diversity based on dietary pattern. However, we observed changes in the abundance of less prominent bacterial phyla based on the DP1 but not DP2. In DP1, the abundance of Verrumicrobia and Fusobacteria varied over the 6-month period,

whereas in DP2, variation in the abundance of Actinobacteria, Bacteroidetes, Firmicutes, Cyanobacteria and Verrucomicrobia were observed. Children falling above the median in DP1 had the least changes at the phyla and genera level, whereas children above the median in DP2 showed the greatest changes. These results suggest that an eating pattern defined by relatively high intakes of high fiber foods such as fruits, vegetables and grains, but lower intakes of starchy foods and juice and sweetened beverages and might be associated with a more stable microbiota. Higher variability in microbiota composition over a 6-months period was observed in a dietary pattern characterized by lower intakes of vegetables, but higher intakes of animal products such as protein and dairy foods. These results could be of significance as less variability in microbiota composition has been associated with greater resistance to pathogen invasion (Ley et al., 2008). We did not find significant differences in intakes of processed foods between the two dietary patterns. This could be in part attributed to the *a priori* selection of foods to include in each food group which was based on previously published methods. For example, French Fries, which are often high in the diet of American children, was grouped together in a group with other refined carbohydrates such as white bread or bagels.

Besides changes in microbial composition, determining variability in microbial metabolites concentration could have important health implications as some short chain fatty acids have been associated with GI health (Macfarlane & Macfarlane, 2012). Herein, we observed an increase in acetate concentrations in children falling above the median in DP1 and an increase in butyrate concentration in children with scores below the median in DP1. In DP2, increases in acetate and butyrate concentrations were observed in children falling above the median. Some of the changes in the VFA concentrations observed herein could potentially be attributed to changes in dietary intake as well as changes in the microbiota composition.

Although not statistically significant, some bacteria known to produce VFA numerically increased in this study include *Bifidobacterium, Faecalibacterium and Ruminoccous*. Previous studies have reported fluctuations in VFA concentrations over the course of the day (Kaczmarek et al., 2017) and in response to dietary interventions (Vanegas et al., 2017); however, to the best of our knowledge no studies have investigated fluctuations in VFA over a longer period of time. SCFA have been suggested to be involved in communication pathways of the microbiota-gut-brain axis and higher SCFA concentrations were reported in fecal samples of children with ASD (Wang et al., 2012). Additionally, one study found significant associations between higher fecal butyrate concentrations and emotional problems (Michels et al., 2016). On the other hand, increased production of SCFA has also been proposed in maintaining colon health (Clausen & Mortensen, 1995). Thus, temporal variability in VFA concentrations could be associated with changes in host responses. In our study no changes in GI health were observed and no information on behavioral aspects was collected. Therefore, the significance of these changes remains to be determined. However, the data presented herein could provide preliminary evidence for future larger studies investigating changes in VFA concentrations in relation to host health.

A novel finding of the current study is the observation of distinct bacterial profiles based on dietary patterns, even though differences in alpha- or beta-diversity as well as VFA concentrations were not observed. Defining a healthy microbiota is important due of the ability of the gut microbiota to affect host health and behavior (Burokas et al., 2015). Establishing a definition for a healthy microbiota is difficult and to date no clear understanding of a "beneficial microbiota" has been established (Linares et al., 2016). However, bacteria thought to be beneficial can exert positive health benefits on the host such as supporting the immune system

and protecting against pathogens. In our study, some of the bacterial genera and phyla that differed between the two groups in DP1 have been implicated to benefit host health. Children in the above median in DP1 had higher levels of Rikenellaceae, Erysipelotrichaceae, Bacteroidetes, *Bacteroides*, *Lachnospira*, *Ruminococcus*, and *Hafnia*, but lower levels of Clostridiaceae, *Bifidobacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus* and *Roseburia*. Even though beneficial effects are associated with higher abundance of *Bacteroides*, such as supporting development of immune tolerance and avoidance of allergy and asthma, some negative associations have also been reported. For example, *Bacteroides* could have negative effects due to its virulence factors causing infection after colonic contamination of abdominal cavity or tissues or GI illness due to production of enterotoxins (Wexler, 2007). Previous studies reported a higher abundance of *Bacteroides* in individuals following a Western-style diet, which is characterized by intakes high in protein and fats similar to intakes for children above the median in DP1 (Wu et al., 2011). Interestingly, we also found that a higher fruit intake was correlated with a higher abundance of *Bacteroides* and Bacteroidetes and intake of refined carbohydrates was negatively associated with the relative abundance of *Bifidobacterium*. Similarly, other studies have also shown that a diet rich in fruit and legumes resulted higher abundance of *Bacteroidetes* (De Filippo et al., 2010). We have previously shown that the abundance of *Ruminoccocus* can negatively predict brain N-acetylaspartate, a marker of neuronal health, concentration through the levels of serum cortisol, which could impact neurodevelopmental disorders (Mudd et al., 2017). Likewise, *Lachnospira* has been found to be enriched in individuals with Inflammatory Bowel Syndrome (IBS), but could also potentially protect against the development of asthma (Claassen-Weitz et al., 2016; Rode et al., 1981). Even though no differences in butyrate concentrations at baseline between the groups in DP1 were observed, one

of the potential benefits of a greater abundance of *Roseburia* could be through the production of butyrate in the colon, which is the preferred substrate for colonocytes and has been associated with prevention of colitis and colorectal cancer (Pryde et al., 2002; Jacobasch et al., 1999). Additionally, *Roseburia* was present in higher abundance in lean compared to obese subjects (Aguirre et al., 2016). Health benefits of *Prevotella* include improved glucose tolerance and improved gastrointestinal health (Monk et al., 2017) and *Prevotella* has been shown to be decreased in children with ASD (Kang et al., 2013). *Bifidobacterium* is generally associated with beneficial effects to human health, including prevention of colorectal cancer, treating gastrointestinal disorders such as diarrhea or IBD or anxiolytic like effects (O'Callaghan & van Sinderen, 2016; Savignac et al, 2015).

In DP2, fewer differences were found between the microbiota composition of children in the above and below median groups. Children above the median had higher fecal *Dorea*, *Eubacterium,* and SMB53, but lower levels in Cyanobacteria and *Phascolarctobacterium* compared to the below median group. Individuals with a higher abundance of *Dorea* were more resistant to *Campylobacter* infection (Kampmann et al., 2016) whereas individuals who became *Campylobacter* positive had higher abundance of *Phascolarctobacterium* (Dicksved et al., 2014). Lastly, *Eubacterium* could potentially inhibit growth of harmful bacteria through its butyrate producing capabilities (Gibson & Roberfroid et al, 1995). Thus, the microbial profile associated with DP2 may be beneficial in protecting against potential pathogenic infections. These results oppose our hypothesis that a dietary pattern higher in animal products will be associated with a less beneficial microbiota. However, processed food such as refined carbohydrates was not different between the groups. Additionally, children above the median in DP2 also had high

intakes of legumes, nuts and sees which are rich in fiber and could potentially offset some of the microbial differences elicited by high intakes of animal products.

Manipulating the GI microbiota has become a promising target for the interventions to treat symptoms of certain diseases, including ASD. Probiotic supplementation has shown some promising results in alleviating some symptoms of ASD (Berding & Donovan, 2017). De Filippo and co-workers hypothesized that a long-term dietary pattern rich in polysaccharides in children living in Burkina Faso shapes the GI microbiota in a fashion that protects against inflammation and noninfectious colonic diseases (De Filippo et al. 2010). Likewise, changing dietary intake for 2 years restored a dysbiotic microbiota in obese individuals (Haro et al., 2017). Herein, we report higher GI severity index scores in the above median group in DP1, which also had higher relative abundance of *Lachnospira* and lower abundance of *Bifidobacteria*. Similarly, higher abundance of *Lachnospira* species and lower abundance of *Bifidobacteria* was reported in individuals with bloating or abdominal pain (Zhang et al., 2015).

This study was limited by the small sample size which did not allow for enough statistical power to assess associations of interest using robust, multiple regression models. The short time between the sample collections is also a limitation. Dietary intake was based on parent self-report using food diaries and food frequency questionnaires, which are susceptible to measurement error and systemic biases in estimate food intake (Willett, 2011). Additionally, our study design only allows for assessment of association and no inferences regarding causality can be made. Therefore, using animal models or human intervention trials in the future will be important in delineating the causality between GI microbiota and host health in h.

Despite these limitations, this is the first study to use the approach of a Principal Component Analysis to define eating patterns and the relationship to microbiota in children. This approach

could provide valuable insight in future studies investigating diet as a moderator for the impact of microbiota on host physiology, cognitive function, and behavior. Additionally, our study contributes to the limited knowledge of temporal microbial variability in children.

In conclusion, the microbiota composition of children aged 4-8 years is distinct based on specific dietary patterns. Furthermore, some dietary patterns may be associated with more stable microbial profile than others. Dietary patterns are important in predicting the long-term composition of the GI microbiota and it has been suggested that an individual's bacterial communities can affect the responses to a specific intervention (Ley et al., 2008). Thus, understanding which dietary patterns are associated with a more stable microbiota can be important in designing therapies and clinical interventions for microbiota-associated diseases.

3.5 Tables and Figures

Table 3.1 Demographic characteristics of all study participants (n=22) at baseline

Characteristic	**Mean ± SD or *n***
Age (years)	5.9±1.3
Gender (n)	
Female	8
Male	14
Race/Ethnicity (n)	
Caucasian	14
Asian	3
Black or African American	3
Hispanic or Latino	2
Early Feeding Practice (n)	
Exclusively Breast-fed	10
Combination with Formula	11
Formula-fed	1
Mode of Delivery (n)	
Vaginal	13
Planned cesarean-section	2
Emergency cesarean-section	7
Mean BMI and Percentile (BMI-for-age)	
Female	17.03±2.5 (85^{th} percentile)
Male	16.4±3.2 (75^{th} percentile)
Nutritional Supplement Use (n)	
Yes	11
No	11

Table 3.2 Factor Loading Matrix

Food Group	Dietary Pattern 1	Dietary Pattern 2
Fish	0.81*	-0.09
Refined Carbohydrates	0.75*	-0.03
Vegetables	0.70*	0.06
Juice and Sweetened Beverages	0.67*	0.02
Protein Foods	0.64*	0.37
Kids Meals	0.52*	0.39
Condiments	0.48*	0.32
Snack and Sweets	0.45*	0.37
Fruit	0.57*	0.47*
Starchy Foods	0.45*	0.61*
Grains	-0.13	0.86*
Dairy	0.17	0.61*
Legumes, Nuts and Seeds	-0.04	0.60*

*Factor loading >0.45 is considered to be a major contributor to the overall dietary pattern; Food groups in which factor loadings are >0.45 for both dietary pattern are assigned to dietary pattern for which food group has highest factor loading contribution; dietary patterns are derived from YAQ data

Table 3.3 Participant characteristics by scores above or blow the median at baseline

Characteristic	Dietary Pattern 1		Dietary Pattern 2	
	Above Median (n=11)	Below Median (n=11)	Above Median (n=11)	Below Median (n=11)
Age	6±1.5	5.8±1.3	5.5±1.4	6.3±1.2
Gender (n)				
Male	8	5	6	2
Female	3	6	5	9
BMI (kg/m^2)	16.5±2.9	16.7±3.1	16.7±3	16.6±3
Race (n)				
Asian	3	0	1	2
African American	1	2	2	1
Hispanic	0	2	1	1
White	7	7	7	7
Current Nutritional Supplement Use (%)	27	27	18	32
GI Severity Index	0.82±0.75	0.36±0.67†	0.15±0.4	1.0±0.77*
Bristol Stool Chart (n)				
Type 2	1	1	0	2
Type 3	6	3	3	6
Type 4	4	7	8	3

Data are expressed as Mean ± SD or *n*; p-values were obtained by Wilcoxon Rank Sum Rest and Chi-Square Test. Values within same dietary pattern and row are significant at *$p<0.05$ and †$p<0.1$
GI, gastrointestinal; BMI, body mass index

Table 3.4 Baseline food and nutrient intakes of participant characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2

	Dietary Pattern 1		**Dietary Pattern 2**	
	Above Median (n=11)	Below Median (n=11)	Above Median (n=11)	Below Median (n=11)
Food Groups Intake (servings per day)	2.9±1	2.1±1.2*	2.8±1.4	2.3±0.9
Fruit	2.4±1.0	1.6±0.6*	1.9±1.2	2.1±0.6†
Vegetables	0.4±0.3	0.5±0.3	0.6±0.4	0.3±0.1*
Legumes, Nuts, Seeds	0.5±0.2	0.4±0.3	0.5±0.33	0.4±0.2
Starchy Foods	0.6±0.4	0.9±0.8*	0.7±0.8	0.8±0.5
Juice and Sweetened Beverages	0.5±0.2	1.1±0.9*	1.0±0.9	0.6±0.4
Grain Foods	1.8±0.8	1.3±0.2*	1.6±0.6	1.6±0.7
Refined Carbohydrates	1.8±0.6	1.2±0.8	1.5±0.8	1.4±0.8
Protein Foods	4.2±1.9	4.0±2.2	5.0±1.9	3.1±1.6*
Dairy Foods	2.3±0.8	1.9±0.8	2.4±0.8	1.7±0.6*
Snack and sweets	0.8±0.4	0.6±0.5†	0.7±0.5	0.7±0.4
Kids Meals	0.4±0.2	0.1±0.1*	0.2±0.2	0.3±0.2
Fish	0.4±0.3	0.3±0.2	0.4±0.3	0.3±0.2
Condiments				
Nutrient Intake				
Cholesterol (mg)	201±84	176±134†	203±123	173±100
Total saturated fatty acids (g)	20±4	21±9	23±8	18±5*
Total Trans Fatty acids (g)	3±1	2±1*	2±1	2±1
Vitamin A (μg)	412±216	465±151*	479±207	397±155
Vitamin C (mg)	98±66	81±53	65±47	113±62*
Calcium (mg)	791±352	861±218	902±253	750±312†
Whole grains (g)	1±1	2±1*	2±1	1±1
Total Dietary Fiber (g)	16±8	15±4	16±8	16±3
Soluble Dietary Fiber (g)	5±2	5±1	4±2	5±1
Insoluble Dietary Fiber (g)	12±6	11±3	11±8	11±3
Pectins (g)	2±1	2±1	2±2	2±1

Table 3.4 (cont.)

Data are expressed as Mean ± SD; p-values were obtained by Wilcoxon Rank Sum Rest and Chi-Square Test; Values within same pattern and row are significant at *p<0.05 and †p<0.1; Only nutrient intake that showed significant difference between the groups are shown; Food groups intake derived from YAQ; Nutrient intake derived from 3-day food record

Table 3.5 Baseline bacterial abundance and VFA concentrations of participants characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2

	Dietary Pattern 1		**Dietary Pattern 2**	
Characteristic	Above Median (n=11)	Below Median (n=11)	Above Median (n=11)	Below Median (n=11)
Bacterial Abundance (% of sequences)				
Family				
Rikenellaceae	4.7±3.4	2.6±2.2†	3.4±2.6	3.9±3.4
Clostridiaceae	0.2±0.1	0.4±0.4†	0.5±0.4	0.3±0.2
Erysipelotrichaceae	0.4±0.3	1.1±1.2*	1.1±	0.44±
Phyla				
Bacteroidetes	56.4±13.5	39.3±21.2*	49.1±19.5	46.6±20.2
Cyanobacteria	0.01±0.02	0.01±0.02	0.003±0.008	0.01±0.02*
Genera				
Bifidobacterium	1.6±2.2	4.2±5.3†	2.3±3.3	3.5±4.9
Bacteroides	42.5±13.2	28.7±19.0*	28.1±19.8	33.1±15.4
Prevotella	3.03±10.0	4.4±8.7†	1.9±6.3	5.5±11.4
Staphylococcus	0.005±0.004	0.02±0.02†	0.01±0.02	0.01±0.02
Streptococcus	0.1±0.1	0.4±0.5†	0.2±0.2	0.3±0.6
Blautia	0.8±0.4	1.5±1.0†	1.2±0.9	1.1±0.8
Dorea	0.9±0.7	1.0±0.6	1.2±0.6	0.7±0.5*
Lachnospira	0.8±0.8	0.3±0.2†	0.5±0.5	0.6±0.8
Roseburia	0.3±0.2	0.8±0.7*	0.7±0.8	0.4±0.2
Ruminococcus	0.44±0.8	0.43±0.4*	0.46±0.36	0.42±0.82
Phascolarctobacterium	0.4±0.7	0.5±1.0	0.24±0.50	0.70±1.5†
Eubacterium	0.07±0.2	0.04±0.03	0.09±0.17	0.02±0.02*
Hafnia	0.03±0.09	0±0†	0.03±0.09	0.0003±0.001
SMB53	1.17±0.9	2.05±2.3	2.06±2.21	1.17±1.06*
VFA Concentration (μmol/g)				
Acetate	104.4±63.7	102.7±69.6	97.4±67.3	109.6±65.5
Propionate	38.9±18.3	38.1±25.3	38.4±23.6	38.6±20.4

Table 3.5 (cont.)

	Dietary Pattern 1		Dietary Pattern 2	
Characteristic	Above Median (n=11)	Below Median (n=11)	Above Median (n=11)	Below Median (n=11)
Butyrate	42.5±35.4	28.6±24.9	30.8±24	40.2±36.7
Isobutyrate	5.7±3.1	4.2±1.7	4.5±2.5	5.4±2.7
Valerate	4.3±2.9	3.1±2.4	3.8±2.7	3.6±2.7
Isovalerate	8.3±4.4	6.2±2.4	6.6±3.6	7.8±3.7

Data are expressed as Mean ± SD; p-values were obtained by Wilcoxon Rank Sum Rest and Chi-Square Test; Values within same dietary pattern and row are significant at *$p<0.05$ and †$p<0.1$; Only bacteria that showed significant difference between the groups of dietary patterns are shown.
VFA, volatile fatty acids

Table 3.6 Relative abundance of bacterial genera in stool at baseline, 6 weeks and 6 months post-baseline of participant characterized as consuming above or below the median in dietary pattern 1 (a) and dietary pattern 2 (b)

a) Dietary Pattern 1

	Above Median			Below Median		
Bacterial genus (% of sequences)	**Baseline**	**6-weeks p.-b.**	**6 months p.-b.**	**Baseline**	**6-weeks p.-b.**	**6 months p.-b.**
Bacteroidetes						
Paraprevotella	0.37±0.93	0.22±0.47	0.15±0.41	0.09±0.21†	0.34±1.06†	0.0008±0.001†
Firmicutes						
Lachnospira	0.76±0.84	0.75±0.6†	0.44±0.53	0.28±0.23	0.64±0.52	0.67±0.95
Fusobacteria	0±0	0.003±0.006	0.01±0.003	0±0*	0.006±0.007*	0.01±0.006*

Data expressed as Mean±SD; *p≤0.05; †p≤0.1; p.b. – post-baseline; Only genera with abundance >0.05% were analyzed

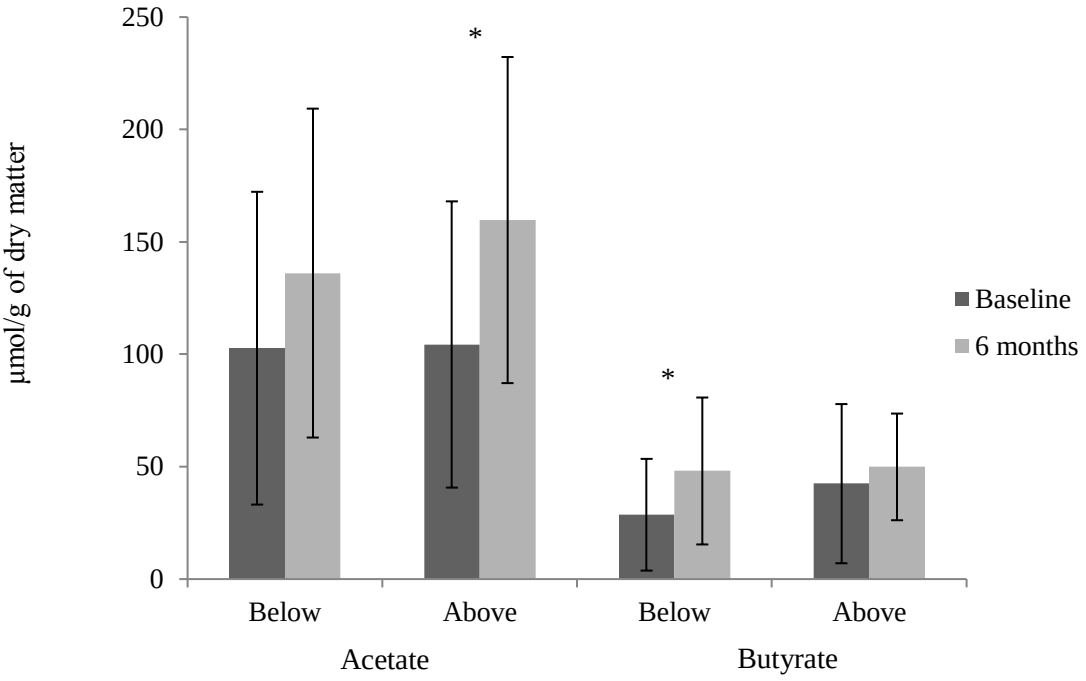
b) Dietary Pattern 2

	Above Median			Below Median		
Bacterial genus (% of sequences)	**Baseline**	**6-weeks p.-b.**	**6 months p.-b.**	**Baseline**	**6-weeks p.-b.**	**6 months p.-b.**
Actinobacteria	2.76±3.39*	0.91±0.54*	3.27±3.29*	3.8±5.1	3.8±6.9	4.1±5.4
Bifidobacterium	2.25±3.32†	0.62±0.36†	2.59±3†	3.49±4.98	3±5.86	3.68±5.3
Bacteroidetes	49.13±19.53*	54.08±11.89*	40.27±14.78*	46.59±20.19	49.07±18.45	51.41±19.68
Firmicutes	40.94±14.93†	40.22±14.82†	54.7±13.12†	41.21±14.62	45.74±14.29	38.99±20.26
Streptococcus	0.2±0.18†	0.08±0.1†	0.12±0.16†	0.31±0.55	0.15±0.16	0.05±0.03
Lachnospira	0.49±0.53	0.76±0.59	0.59±0.91	0.55±0.77*	0.63±0.59*	0.5±0.55*
Roseburia	0.71±0.82*	0.31±0.31*	0.44±0.32*	0.39±0.21	0.5±0.44	0.36±0.29
Cyanobacteria	0.004±0.008	0.02±0.06	0.09±0.25	0.02±0.02*	0.008±0.02*	0.005±0.007*
Verrucomicrobia	6.39±13.66	3.42±6.5	1.07±20.3	6.75±9.84†	0.37±0.61†	4.76±9.75†

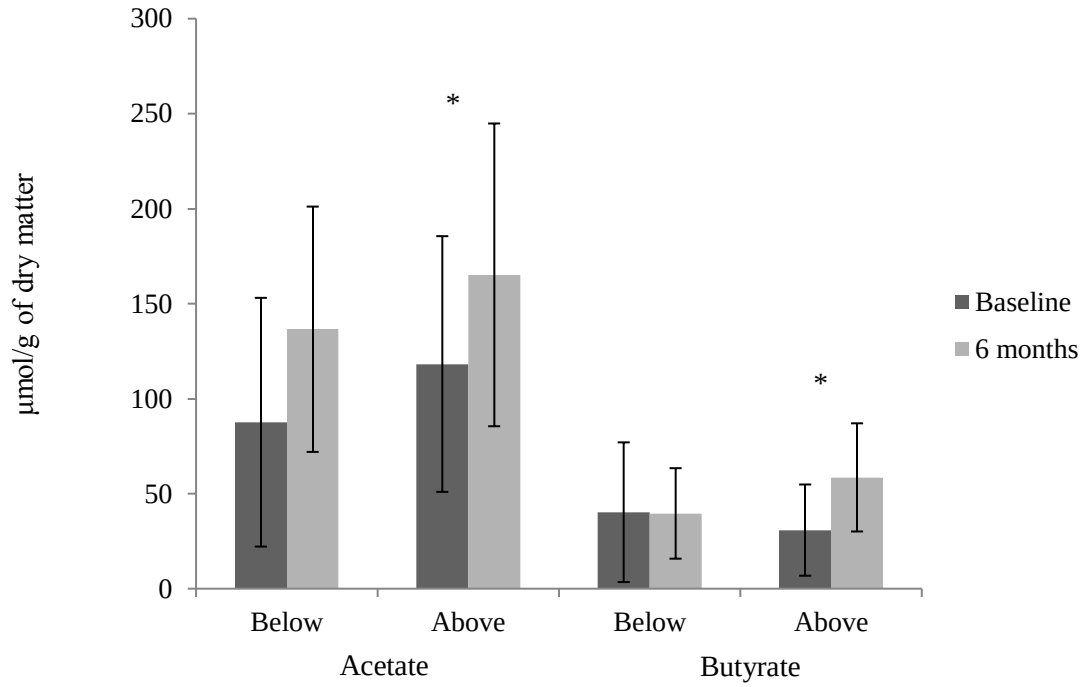
Data expressed as Mean ± SD; *p≤0.05; †p≤0.1; p.b. – post-baseline; Only genera with abundance >0.05% were analyzed

Figure 3.1 Volatile fatty acid concentrations at baseline and 6 months post-baseline

a) VFA concentrations at baseline and 6 months based on Dietary Pattern 1



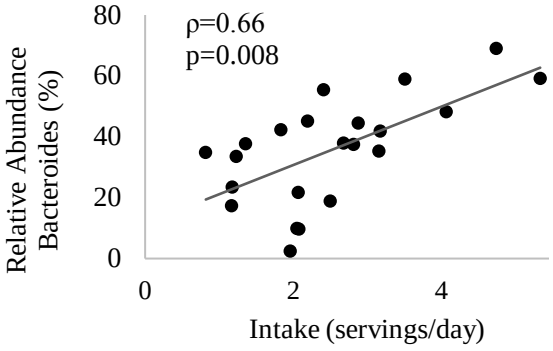
b) VFA concentrations at baseline and 6 months based on Dietary Pattern 2.



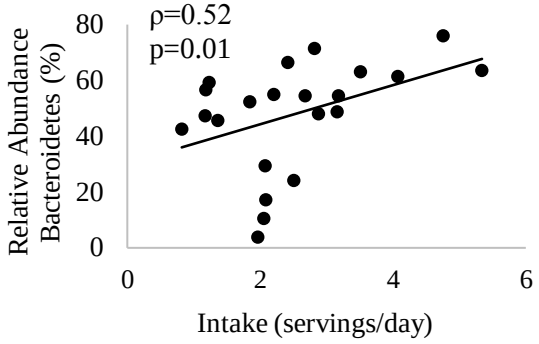
*p<0.05 indicates a difference between baseline and 6 months. VFA, volatile fatty acids

Figure 3.2 Correlation between intake of specific food group and abundance of bacteria

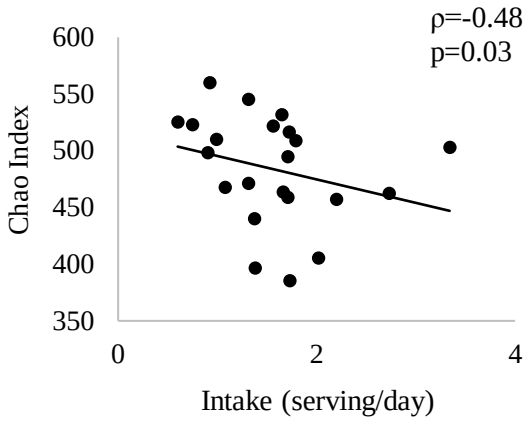
a) *Bacteroides* and intake of servings of fruit



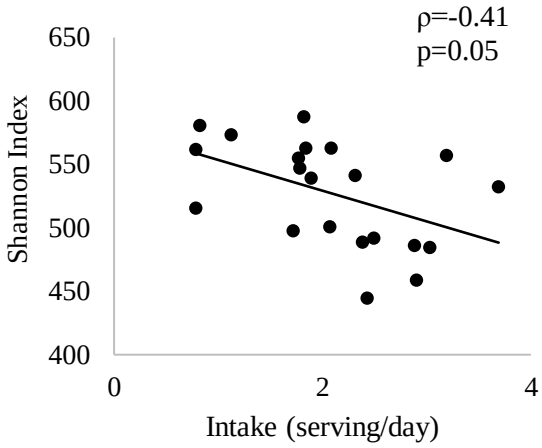
b) *Bacteroidetes* and intake of servings of fruit



c) Chao 1 Index and intake of refined carbohydrates



d) Shannon Index and intake of snack and sweets



Spearman Correlation coefficient for selected food groups derived from the YAQ and bacteria showing strongest correlation

CHAPTER 4

Differences in Microbiota Composition and VFA Concentrations Between Children with ASD and Unaffected Controls and Relationship to Symptom Severity[3]

Abstract

Background/Aims: An increasing number of studies report differences in the fecal microbiota composition and volatile fatty acids (VFA) between children with Autism Spectrum Disorder (ASD) and unaffected controls. Additionally, increasing evidence shows that specific bacterial taxa can influence some symptoms of ASD.

Methods: We investigated the fecal microbiota composition in children with ASD (n=26) and unaffected controls (n=32) aged 2-7 years. Fecal samples were collected and microbiota composition was analyzed using 16S rRNA sequencing and Real-Time qPCR. VFAs were analyzed using gas chromatography. Social deficit scores in children with ASD were measured using the Pervasive Developmental Disorder Behavior Inventory-Screening Version (PDDBI-SV). Generalized linear mixed models were used to analyze differences in microbiota and VFA concentrations between the groups. Regression analysis was performed to investigate whether individual microbiota could predict social deficit scores.

Results: Overall, β-diversity differed between the groups and observed OTUs were higher in children with ASD. Regarding the microbiota abundances, differences on the phyla, family, order and genera level were observed. Namely, children with ASD had higher levels of Firmicutes, Clostridiales, Clostridiaceae, Peptostreptococcaceae, Coriobacteriaceae, *Clostridium*, *SMB53*, and *Roseburia* but lower levels of Bacteroidetes, Rikenellaceae, *Butyrivibrio*, *Faecalibacterium*, *Dialister*, and *Bilophila* compared to controls. Furthermore, higher

[3] Berding K, Donovan SM. Diet and Feeding Behavior are Related to Microbiota Composition in Children with Autism Spectrum Disorder. *Front Neurosci*. 2018. *Under Review.*

concentrations of acetate, propionate and butyrate were detected in children with compared to controls. Lastly, we found that Peptostreptococcaceae and *Faecalibacterium* predicted social deficit scores in children with ASD as measured by the PDDBI-SV.

Conclusion: In conclusion, this study contributes to the growing knowledge of microbial dysbiosis in children with ASD and provides new evidence for the impact of individual bacterial taxa on some symptoms of ASD.

4.1 Introduction

In recent years, the involvement of the gastrointestinal (GI) microbiota in cognitive and neurodevelopmental disorders, such as anxiety, depression and Autism Spectrum Disorder (ASD), has become increasingly recognized as a potential pathway of affecting symptom manifestation. In ASD, aberrations at the phyla and genus level between children with and without ASD have been described. For example, several studies have shown higher abundance of Bacteroidetes and Proteobacteria, but lower abundances of Firmicutes, Actinobacteria and Verrucomicrobia (Finegold et al., 2010; Kang et al., 2013; De Angelis et al., 2013). On the genera level significant reductions in the relative abundances of *Prevotella, Coprococcus, Enterococcus, Lactobacillus, Streptococcus, Lactococcus, Staphylococcus, Ruminococcus* and *Bifidobacteria* in children with ASD compared to non-affected controls have been reported (Kang et al., 2013; De Angelis et al., 2013; Wang et al., 2011). Conversely, the genera *Clostridium, Sutterella* and *Desulfovibrio* were increased in children with ASD (Finegold et al., 2010; Kang et al., 2013; Parracho et al., 2005). Furthermore, associations between the abundance of specific bacteria and ASD symptom severity have been described. A lower ratio of Bacteroidetes-to-Firmicutes and a higher abundance of *Clostridium* and *Desulfovibrio* were

positively associated with severity of ASD symptoms (Tomova et al., 2015). Besides differences in the fecal microbiota, deviations in microbial products between children with ASD and unaffected controls have also been observed. Previous studies found elevated Volatile Fatty Acid (VFA) concentrations in stool, urine and serum of children with ASD (Wang et al., 2012) and administration of propionate has been shown to induce autism-like behaviors in rodents (MacFabe et al., 2011), suggesting that some VFA could be a way of communication between the GI microbiota and the brain in ASD.

Even though emerging evidence suggests that the GI microbiota might be involved in ASD symptomology, more carefully controlled studies are necessary to define an "ASD microbiome". Effects of probiotic, antibiotic or medication use, specialty diets or presence of functional GI disorders on the GI microbiota composition are often overlooked. Likewise, studies describing the relationship between specific GI bacteria taxa and ASD symptoms are limited. Therefore, the goal of this study was to investigate the microbiota composition of children with ASD and unrelated controls, controlling for recent probiotic, antibiotic or medication use, specialty diets and functional GI disorders. Additionally, food diaries were collected to control for the impact of nutrition on the GI microbiota composition. Lastly, the relationship between GI microbiota and ASD symptoms was investigated. We hypothesize that children with ASD will have a different microbiota composition and VFA concentrations compared to unaffected controls and that individual bacterial taxa will predict the severity of some symptoms of ASD.

4.2 Materials and Methods

Participants and Questionnaire

Children diagnosed with ASD (ASD) between 2 and 7 years-of-age (n=26) were recruited from different sites across Illinois, Indiana, Ohio, Missouri, Kentucky, and Iowa between April 2016 and October 2017. Age- and sex-matched unrelated control subjects (CONT) were recruited in the Champaign-Urbana area. All subjects were free of functional digestive disorders, had not used antibiotics, probiotics or prebiotic in the 3 months prior to enrollment in the study, did not take any medication and did not follow any special diet (e.g., gluten-free/casein-free diet). Parents completed an online questionnaire including questions regarding their child's age, gender, early feeding practices, mode of delivery, nutritional supplement use, gastrointestinal symptoms, height and weight. Parents also answered questions on their perception of child's feeding problems based on information obtained from previously published results ("Do you consider your child to be a picky eater" (Taylor et al., 2015), "Does your child have a repetitive eating pattern (i.e., likes to eat the same foods)" (Cornish, 1998) and "Does your child currently include more than 20 foods in his or her diet" (Nadon et al., 2011; Cornish, 1998). The height, weight and BMI of the participants were converted to percentiles according to the CDC growth charts for both male and female participants. Participants provided oral assent and their legal guardians provided written consent in accordance with the ethical standards of the Institutional Review Board of the University of Illinois at Urbana-Champaign.

Fecal Sample Collection, DNA Extraction and 16S rRNA Sequencing and Analysis

For each subject, freshly-voided morning stool samples were collected for the analysis of the fecal microbiota and VFA concentrations. Stool samples were collected into a plastic commode specimen collection system (Fisher Scientific, Waltham, MA) or directly from the diaper. Parents were provided with gloves and a sterile spoon to transfer ~5 to 10 g of freshly-

voided fecal material into a sterile 50 mL conical tube. All samples were immediately placed in the participant's freezer (-20°C) until transported to the laboratory. All samples were stored in the laboratory at -80°C prior to analysis.

Microbial DNA was extracted from stool using a bead beating method followed by a combination of QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) and the FastPrep-24 System (MP Biomedicals, Carlsbad, CA). Briefly, 180-220 mg of stool were weighed into a Lysing Matrix E tube and 1 mL of Inhibit EX buffer was added to the sample. The tubes were shaken using the FastPrep-24 system, before being incubated at 95°C for 5 minutes. Samples were centrifuged and the supernatant was added to 25 μL of Proteinase K. After adding 400 μL Buffer AL, the mixture was incubated at 70°C for 10 minutes. 400 μL of ethanol were added to the lysate. The lysate was then applied to the QIAmp spin column and centrifuged. 500 μL of Buffer AW1 and AW2 were then added to the column which was then centrifuged again. Lastly, 180-220 μL Buffer ATE was added to the column to dilute the DNA. DNA concentration was measured using the Nanodrop.

Amplification of the V3 to V4 regions (ca. 430 bp) of 16S rRNA gene was performed using dual-index paired-end sequencing approach using primers F357 and R805 (Klindworth et al., 2013). The AccuPrime™ Taq DNA Polymerase System (Life Technologies, Grand Island, NY) was used for PCR amplification in a DNAEngine (Bio-Rad, Hercules, CA). The amplicons were mixed in equimolar concentration and sequenced at the W. M. Keck Center at the University of Illinois, Urbana-Champaign. Paired-end sequencing (2 x 300 bp) was performed with an Illumina MiSeq (Illumina, Inc., San Diego, CA) using version 3 chemistry.

The 16S rRNA sequences were processed and analyzed using the QIIME 1.9.1 bioinformatics package (Caporaso et al., 2010; Bokulich et al., 2013). Sequences were

demultiplexed and clustered into operational taxonomic units (OTUs) using closed-reference OTU picking with default parameters against the Greengenes 13_8 reference OTU database at a 97% similarity level. Singletons and OTUs with an abundance lower than 0.005% were removed prior to rarefying to a sampling depth of 49,446 sequences per sample for subsequent analysis. α- and β- diversity were calculated using QIIME. Taxonomy summary was performed using the core diversity script in QIIME.

Real-time Polymerase Chain Reaction

RT qPCR specific bacteria. Bacterial genomic DNA was analyzed for total bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *Prevotella*, *Clostridium perfringens* and *C. difficile* using primer/probe sequences and annealing temperatures shown in **Table 4.1**. These specific bacterial genera were chosen based on the ability to produce VFA or propionate (*Prevotella spp*.) as well as their notion as beneficial and harmful bacteria (Louis et al., 2007; Macfarlane & Macfarlane, 2012).

Real-time qPCR was performed in Quant Studio 6 and 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using SYBR Green assays. Each reaction contained 5μL of 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 1μL of bovine serum albumin (New England Biolabs, Ipswich, MA) at 1 mg/mL (final concentration 100μg/ml), 0.5 μmol/L of each primer and 1μL of water. 8μL of PCR mix and 2 μL of sample containing 10 ng of DNA (*Lactobacillus* spp., *Bifidobacterium* spp., *Prevotella*, *C. perfringens, C. difficile*) and 0.5ng of DNA (total bacteria) were plated on a MicroAmp Optical 384-well reaction plate (Applied Biosystems).

The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, primer-specific annealing temperature for 20 seconds and 72°C for 45 seconds. Standard curves ($5x10^0$ – $5x10^7$ gene copies per reaction) were prepared using purified PCR 4 TOPO-TA plasmids (Life Technologies, Carlsbad, CA) containing 16S rRNA genes of *Eubacterium hallii* 27751 (total bacteria), *Lactobacillus rhamnosus* 53103 (*Lactobacillus* spp.), *Bifidobacterium longum* 15007 (*Bifidobacterium* spp.*), Prevotella ruminicola* 19189 *(Prevotella), C. perfringens* 13124 (*C. perfringens*) and *C. difficile* 9689 (*C. difficile*). Data was analyzed using QuantStudio Design Analysis Software 1.3 (Thermo Fisher Scientific). Results are presented as the $\log_{10}$ of the number of copies per gram of wet sample.

RT qPCR for mmDA and BCoAT. In addition, bacterial genomic DNA was analyzed for the detection of bacterial genes present in the methylmalonyl-CoA decarboxylase (mmdA) gene in the succinate pathway of propionate production and the butyryl-CoA:acetate CoA acyltransferase (BCoAT) gene in the butyrate production pathway. These pathways appear to be the most abundant pathways used for propionate and butyrate formation by the bacteria inhabiting the human gastrointestinal tract (Louis et al., 2010; Reichardt et al., 2014). Sample preparation and analysis was performed as described above. The primer/probe sequences and annealing temperatures are shown in **Table 4.1**. Briefly, real-time qPCR was performed with 5µL of 2x Power SYBR Green PCR Master Mix, 0.5 µmol/L of each primer and 1µL of water and bovine serum albumin at 1 mg/mL. 8µL of PCR mix and 2 µL of sample containing 20 ng DNA were plated on a MicroAmp Optical 384-well reaction plate. mmdA and BCoAT genes were quantified in parallel with a universal 16S rRNA gene. Data was analyzed using

QuantStudio Design Analysis Software 1.3. Results are presented as mmdA or BCoAT over total 16S rRNA.

VFA Analysis

Sample preparation for VFA analyses was performed as follows: fecal material (0.1 g) was weighed and mixed with 0.4 mL of 3.25% m-Phosphoric acid solution in a 2 mL microcentrifuge tube. Samples were vortexed to disperse the sample in solution. After an incubation time of 30 min, samples were frozen over night at -20°C. The next day, the samples were thawed and centrifuged in microfuge tubes at 13,000×g for 10 min. The supernatant was transferred into gas chromatography vials.

The VFA concentrations were analyzed by a Hewlett-Packard 5890A Series gas chromatograph and a glass column (180 cm 3 4 mm i.d.), packed with 10% SP-1200/1% H3PO4 on 80/100 mesh Chromosorb WAW (Superlco). Oven temperature, detector temperature, and injector temperature were set at 1258°C, 1758°C, and 1808°C, respectively. VFA production was calculated as VFA concentrations of substrate-containing tubes minus the VFA content of blank tubes divided by substrate weight expressed on a dry matter basis (Li et al., 2012).

ASD Symptoms Severity Assessment

Parents were asked to complete the Pervasive Development Disorder Behavior Inventory Screening Version (PDDBI-SV) (PAR, Inc., Lutz, FL) in order to assess the social deficit symptom severity. The PDDBI-SV was developed for clinicians as a tool to quickly screen children at risk for ASD, but can also be used to assess ASD symptom severity for research purposes (Cohen, 2011). The PDDBI-SV is an 18 item parent questionnaire developed for

children ages 18 months to 12.5 years. It is an abbreviated version of the PDD Behavior Inventory (PDDBI) which is a reliable and validated assessment tool. Nine questions from the Social Pragmatic Problems domain and nine questions from the Social Approach Behaviors from the original PDDBI were used to develop the screening version. The PDDBI-SV produces one composite score, the Social Deficit (SOCDEF) score. Each question has five response options that are rated on a Likert scale. In the Social Pragmatic Problem domain the answers a scored according to 0 "Does not show behavior", 1 "Rarely shows behavior", 2 "Sometimes/Partially shows behavior", 3 "Usually/typically shows behavior' and "Don't understand". In the Social Approach Behaviors domain items are reverse-scored The answers are scored by summing the ratings and yield an overall T score for the severity of ASD symptoms. The T score is grouped into six levels of severity. Level 1 (unlikely) range from <22 to 30; level 2 (borderline) ranges from 21 to 35, level 3 (mild) ranges from 36 to 40, level 4 (moderate) ranges from 41 to 59, level 5 (severe) ranges from 60 to 69 and level 6 (extreme) ranges from 70 to >80. A T score of 50 is defined as typical of children with ASD. As the SOCDEF score increases, social communication skills worsen and challenging behaviors increase.

Assessment of Gastrointestinal Symptoms and Stool Consistency

The severity of GI symptoms was assessed for constipation, diarrhea, stool smell, flatulence and abdominal pain in an adapted version of the GI Severity Index. This version has previously been used in a study investigating GI health in children with ASD (Adams et al., 2011). Parents assessed their child's severity of constipation, diarrhea, stool smell, flatulence and abdominal pain on a scale from 0 to 2. Scores for each item were summed to determine an overall severity score.

Additionally, average stool consistency was assessed using the Bristol Stool Chart (Lewis & Heaton, 1997). The Bristol Stool Chart categorizes the stool into seven categories, ranging from separate hard lumps (Type 1) to liquid consistency with no solid pieces (Type 7). The Bristol Stool chart also allows for classification of presence of constipation (Type 1) and diarrhea (Type 7).

3-Day Food Diary

To monitor short-term nutrient intake prior to fecal sample collection, a 3-day dietary food record was completed the 3 days prior to sample collection. The parents were asked to report all types and quantities of food and beverages consumed by their children during the three-day (two week days, one weekend day) period prior to each fecal sample collection. Parents were trained by a member of the research team on how to record their child's meal on the food record. Additionally, parents were provided with a detailed description of how to keep a food record. After collection of the food record a member of the research team followed up with the parent if additional detail on food consumption was needed. The food diary data were analyzed using the Nutrition Data System for Research (NDSR, Minneapolis, MN, 2014) software to assess nutrient intake and for comparison to recommended intakes (i.e. Recommended Daily Allowance, Adequate Intake). In addition, information on food intolerances, food avoidance and nutritional supplement use, including pre- and probiotics, and medical prescriptions was collected in the online questionnaire.

Statistics

All data were analyzed using SAS 9.4 (SAS Institute, Cary, NC). Descriptive statistics (means and frequencies) were generated for all demographic and epidemiologic variables of study participants. Differences in categorical variables were analyzed for significant differences using the Fischer's Exact test.

Differences in microbial community structure were evaluated with principal coordinate analysis (PCoA) and permutational multivariate analysis (PERMANOVA) of variance using weighted and unweighted UniFrac distance in the QIIME software. Linear Discriminant Effect size (LEfSe) analysis was used to detect differentially abundant microbial taxa between ASD and control subjects (Segata et al., 2011). LEfSe uses a non-parametric factorial Kruskal-Wallis test with an α-level of 0.05 to determine differential distribution of taxa among the groups. Then a LDA model is build using identified microbial taxa and effect size for the significantly different taxa is estimated. The threshold on the logarithmic LDA score for discriminative feature was set to 3.0. A higher LDA score for a taxon corresponds to a more significant relationship. LEfSe was performed using the website http://huttenhower.sph.harvard.edu/galaxy .

Differences between the groups in α-diversity (Chao1, Shannon Index and observed OTUs), relative abundance of individual phyla, families, orders and genera, and VFA were analyzed using mixed models. Factors known to influence the microbiota composition including age, gender, BMI, season of sample collection and dietary fiber intake were included as co-variates in each model. Model fit was assessed using the chi-square-to-df ratio. Values <2 were indicate an appropriate model fit.

Multiple Linear Regression was utilized to investigate whether individual microbiota or VFA can predict SOCDEF scores. Model adequacy was tested for each model including the

independent variables identified through stepwise regression. Variance Inflation Factor (VIF) was calculated to determine whether multicollinearity is present in the model. Correlations between the independent variables were tested and normality of the residuals was measured using Shapiro-Wilk. Variance homogeneity was tested by SPEC test and the Durbin-Watson test was applied to test for residual correlation. Cook's D was calculated for each model to determine the influence of individual observations. Variables with a Cook's D greater than 0.5 were excluded from the model. The Mallow's Cp value was calculated and plots were reviewed to assess the goodness of fit for each model.

Pearson and Spearman correlation was performed to analyze the relationship between VFAs and SOCDEF scores. Data are expressed as mean ± SD or median (interquartile range (IQR)). Level of significance was set at $p \leq 0.05$ and $p \leq 0.10$ was considered a trend.

4.3 Results

Enrollment and Baseline Samples Collected

Initially, 34 children were enrolled in the CONT group and 41 ASD group. However, samples were collected from 32 (94%) CONT subjects and 26 (63%) ASD subjects. After enrollment in the study, parents of subjects were contacted via E-mail or phone multiple times to confirm their interest in collecting baseline samples. After contacting the parents 3 times, the child was excluded from the study.

Demographics of Study Population

The study population demographics for each group are shown in **Table 4.2**. The mean age of the ASD group was 4.1±1.6 years and the control group 3.9±1.3 years and did not differ

significantly between the groups. Gender and race/ethnicity did not differ between the groups. Most participants were male (ASD: 18 male vs. 6 female; CONT 19 male vs. 13 female) and most participants self-identified as Caucasians (ASD 68%, CONT 63%). No differences between the groups were noted in parental marital status, annual income or health care coverage. In the CONT group, parents had higher levels of education (p=0.001) compared to parents of the ASD group. The mean BMI for female participants in the ASD group was 15.1 (50^{th} percentile) and in the CONT group was 16.8 (82^{nd} percentile). The mean BMI for male participants in the ASD group was 16.8 (70^{th} percentile) and in the CONT group was 16.3 (75^{th} percentile). In the CONT group, 53% of the parents reported that the child was taking a nutritional supplement, whereas only 23% of parents reported nutritional supplement use in the ASD group. Most participants were born between 37-42 weeks of gestation. In the CONT group 75% were born vaginally compared to only 42% in the ASD group. Likewise, participants in the CONT group were more likely to be exclusively breast-fed (p=0.03) compared to the ASD group. In both groups, most parents denied frequent use of antibiotics early in life. Regarding their eating behavior, children with ASD were less likely (p=0.0007) to have more than 20 foods in their diet compared to the CONT group. Likewise, parents of over half of the children in the ASD group (57%) identified their child as having a repetitive eating pattern compared to 40% of the CONT group; however, this was not statistically different.

Additional characteristics of children with ASD are shown in **Table 4.3**. The average SOCDEF T score was 52 which is categorized into the level 4 (out of 6) moderate symptoms category. The average age of parents noticing changes in their child's behavior was 1.4 years and the average age of diagnosis was 2.4 years. About 70% of children in the ASD group are receiving some form of behavior therapy (e.g., Applied Behavior Analysis, Speech therapy,

occupational therapy), about 65% report to have sensory issues and 55% are using some form of Complementary and Alternative Medicine (e.g., Mind Body Medicine, Nutritional Supplements).

GI Severity and Stool Consistency

Presence of GI symptoms was reported in 69% of children with ASD and 46% of unrelated control children. GI symptoms severity was about twice as high (p=0.002) in the ASD group compared to CONT. Specifically, children with ASD had higher scores for stool smell (p=0.006); flatulence (p=0.04) and abdominal pain (p=0.07).

Likewise, stool consistency measured by the Bristol stool chart differed significantly (p=0.05) between the groups. In the ASD group, about one third of the children had harder type stools (Type 1-3), about half have normal stool (Type 4) and one fifth had softer type stools (Types 5-7). In the CONT, one half of the children had harder type stools and the other half had normal stools. GI symptom severity was not associated with SOCDEF scores. The results are shown in **Table 4.4**.

Microbiota Composition

16S rRNA sequencing: Principal Coordinate Analysis (PCoA) of weighted and unweighted UniFrac are shown in **Figure 4.1**. PERMANOVA analysis indicated that overall bacterial communities differed between ASD and CONT group (p=0.02) based on unweighted UniFrac, but not on weighted UniFrac distance. α-diversity tended to differ between the groups when measured as observed OTUs (p=0.08). There were no differences on other measures of α-diversity (Chao 1 Index, Shannon Index, Simpson Index) between the groups (**Table 4.5**).

To identify which specific bacteria differed between the groups, the sequences were classified against the Greengenes Database. At the phyla level (**Figure 4.2**), children with ASD had a lower abundance of Bacteroidetes (p=0.07) but higher abundance of Firmicutes (p=0.03) compared to CONT. Additionally, the abundance of Clostridiales (p=0.07) was higher, whereas the abundance of Streptophyta (p=0.08) was lower in children with ASD compared to CONT. On the family level, children with ASD had significantly higher abundance of Coriobacteriaceae (p=0.04), Clostridiaceae (p=0.07) and Peptostreptococcaceae (p=0.05), but a lower abundance of Rikenellaceae (p=0.005) compared to CONT children. On the genus level, increased abundances of *Clostridium*, SMB53, and *Roseburia*, but decreased abundances of *Butyrivibrio*, *Faecalibacterium*, *Dialister* and *Bilophila* were observed in children with ASD. Abundance of all bacteria can be found in **Table 4.6.**

LEfSe Analysis: To identify taxa that were most differentially abundant between the groups, LEfSe was applied to assess the effect size of each enriched taxon. The most abundant taxa (LDA score >3) in children with ASD were *Prevotella*, *Collinsella*, *Megamonas* and *Clostridium*, whereas Rikenellaceae was most enriched in the CONT group. (**Figure 4.3**).

qPCR results: The abundances of total bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *Prevotella*, *C. perfringens* and *C. difficile* are shown in **Figure 4.4**. The abundances of *Bifidobacterium* spp. (p=0.04) and *C. perfringens* were higher (p=0.009) in the CONT group compared to the ASD group. The density of total bacteria, *Prevotella* spp., and *Lactobacillus* spp. did not differ between the groups. *C. difficile* was only detected in one sample in the ASD (log_{10} gene copy number: 5.42) and two samples in the CONT group (log_{10} gene copy number: 6.22) (detection limit: 5.24 in Log scale). In CONT children, a trend for greater relative BCoAT (p=0.09) and mmDA (p=0.07) gene was observed compared to ASD children

VFA Concentration

Higher concentration of acetate (p=0.02), propionate (p=0.04) and butyrate (p=0.03) were observed in the ASD group compared CONT (**Figure 4.5**). There was no statistical significant difference in the concentrations of valerate, isovalerate and isobutyrate.

Microbiota Predicts SOCDEF Score

In order to understand the relationship between the microbiota and metabolic products, we applied multiple linear regression to analyze whether individual microbiota or microbial products could predict the SOCDEF scores.

Due to the high number of variables, we used stepwise regression to assist in identifying potential independent variables for predicting SOCDEF scores. For independent variables to stay in the model entry and stay levels were 0.05 and 0.1, respectively. α-diversity, relative concentration of BCoAT and mmDA as well as VFA concentrations did not reach the significance level and, thus, were not included in the model. With regard to bacterial family, order, phyla and genera, Peptostreptococcaceae, *Lactobacillus* and *Dialister* and *Faecalibacterium* were identified as significant predictors for SOCDEF score.

Model adequacy was tested for each model including the independent variables identified through stepwise regression. *Lactobacillus* and *Dialister* reached a VIF of greater than 2 and, thus, were excluded from the model. Independent variables were not correlated, and all residuals

were normally distributed at $p<0.05$. Variances were homogenous as tested by SPEC test and the Durbin-Watson test showed no residual correlation. A summary of the fitted models can be found in **Table 4.7**. Age, gender, season, height, weight and BMI were included as co-variates in the model. After quality control, the remaining microbiota in the model, namely Peptostreptococcaceae and *Faecalibacterium* produced an adjusted R^2 of 0.36 ($F(5,18)=5.96$; $p=0.009$) for the prediction of SOCDEF score. Thereby, Peptostreptococcaceae ($\beta=0.33$) and *Faecalibacterium* ($\beta=0.63$) positively predicted SOCDEF scores.

GI Symptoms and Stool Consistency are Related to Microbiota Composition

Association between bacterial taxa and GI symptom severity and stool consistency are shown in **Table 4.8**.

In children with ASD, positive correlations were observed between GI symptom severity and abundance of Firmicutes, Bacteroidales, Rikenellaceae, Clostridiaceae, *Methanobrevibacter*, *Butyricimonas*, *Enterococcus* and *Holdemania*, whereas negative correlations were observed between GI symptom severity and abundance of Bacteroidetes, Cyanobacteria, Streptophyta, and Bacteroides. Differences in microbiota composition based on stool consistency were also observed. Children with ASD and harder (Type 1-3) stool consistency had higher abundance of *Holdemania* ($p=0.01$) compared to children with normal (Type 4) and softer (Type 5-7) stools.

In CONT, higher levels of *Alistipes* ($p=0.05$) were observed in children with harder stool types, Streptophyta ($p=0.05$) was higher in children with normal stool types, and *Dialister* ($p=0.01$) was lowest in children with harder stools. Negative correlations were observed between

Streptococcus, Lachnospiraceae, *Blautia*, and *Coprococcus* and GI severity symptoms and a positive correlation between *Bilophila* and GI severity symptoms.

4.4 Discussion

An increasing number of studies are aimed at deciphering the relationship between the GI environment and ASD symptomology, specifically focusing on the GI microbiota composition. Here, we report differences in the β-diversity as well as observed OTUs between children with ASD and unaffected controls. Additionally, variation in the abundance of bacteria at the phyla, family, order and genus level was observed consistent with previously published literature. Interestingly, the abundances of Peptostreptococcaceae and *Faecalibacterium* were identified as significant positive predictors for SOCDEF score. Furthermore, children with ASD had higher total GI symptoms severity scores. These findings contribute to the growing body of evidence that the microbiota of children with ASD differs from unaffected controls and could potentially affect severity of symptoms associated with ASD.

Children with ASD commonly present with GI symptoms which include chronic diarrhea, abdominal discomfort, constipation or food intolerance and some of these symptoms may contribute to behavioral symptoms (Coury et al., 2012; Adams et al., 2011a). Although higher scores of GI symptoms were found herein, (Adams et al., 2011a, Horvath et al., 1999), no correlations between GI symptom severity and social deficits scores were observed. One explanation for the lack of association could be the relatively low level of GI symptom severity in this study population. The overall score of GI symptom severity of children in this study was lower in comparison to other studies using the same GI symptom scale. Specifically, Adams et al. reported GI symptoms severity at 3.9 out of 6-point scale, whereas children in this study

averaged a severity index of 2 on the same 6-point scale (Adams et al., 2011a). Some of this discrepancy might be explainable by the difficulty of obtaining exact GI symptom severity due to communication deficits in children with ASD (de Theije et al., 2011). A lack of association between GI and ASD symptoms could stem from the PDDBI-SV scale only assessing social deficit symptoms but not the severity or presence of repetitive behaviors. This scale was chosen due to the young age of some participants and the lack of other reliable tools to measure ASD symptoms in children under the age of 4. Thus, other behaviors associated with ASD might show stronger relationships with GI symptoms.

The abundance of specific microbiota in correlation with the presence of GI symptoms has been discussed in the literature. For example, *Sutterella* and *C. perfringens* was mostly increased in children with ASD and concurrent GI symptoms and constipation has been associated with higher levels of Escherichia/Shigella and Clostridium cluster XVIII (Strati et al., 2017; Adams et al., 2011a). Here, positive correlations between GI symptom severity and the abundance of Firmicutes, Bacteroidales, Rikenellaceae, Clostridiaceae, *Methanobrevibacter*, *Butyricimonas*, *Enterococcus* and *Holdemania* were observed, whereas negative correlations were found between GI symptom severity and the abundance of Bacteroidetes, Cyanobacteria, Streptophyta, and *Bacteroides.* Some of these taxa have been associated with GI symptoms in human studies. For example, *Methanobrevibacter*, a methane producing bacteria, has been associated with flatulence in humans and may be related to pathogenesis of some digestive tract symptoms (Seo et al., 2017; Pimentel et al., 2012).

In addition to GI symptoms, stool consistency also provides information on an individual's GI health. A previous study assessing stool consistency in children with ASD showed that 29% of children had loose or mushy stools (Levy et al., 2007). Overall, the stool

consistency differed between the groups and about half of the children in the ASD group reported harder or softer stools, indicating the potential presence of digestive problems in this group. Furthermore, children with ASD and harder stools also tended to have higher abundance of *Holdemania*. Previous studies demonstrated that bacterial richness decreased with decreasing firmness of the stool which could partly be explained by the changes in transit time and thus availability of food sources to the microbiota (Vandeputte et al., 2015). Other studies found more abundant *Prevotella* in subjects with looser stools, whereas the Ruminococcaceae-Bacteroides group was more prominent in subjects with harder stools. Additionally, abundances of *Methanobrevibacter*, *Akkermansia*, *Oxalobacter* and *Butyricimonas* increased with increased stool firmness, whereas *Bacteroides* abundance increased with more loose stools (Vandeputte et al., 2015).

The microbiota-gut-brain axis has gained increased attention in recent years as a way to describe the interaction between the GI microbiota and brain function. Microbial dysbiosis has been identified in ASD and other psychological and psychiatric diseases, including depression and anxiety, even though some studies do no support the hypothesis that the GI microbiota might be involved in ASD symptomology (Gondalia et al., 2012). Differences in bacterial diversity as well as in fecal VFA concentrations between children with ASD and unaffected controls have been reported (De Angelis et al., 2013; Tomova et al., 2015; Son et al., 2015). Recently, other species in the GI tract (e.g., fungi) were investigated for their ability to impact some symptoms of ASD (Strati et al., 2017) and interest in the microbiota composition of the proximal GI tract in children with ASD is rising (Kushak et al., 2017). In this study, measures of β-diversity as well as observed OTUs were significantly different between the groups. There are limited reports on differences in β-diversity between children with ASD and unaffected controls and results do not

indicate a clear trend yet (Son et al., 2015; Strati et al., 2017). Associations between β-diversity and behavior (sociability level, high intensity pleasure and activity level) in children without ASD have been described (Christian et al., 2015). Here, we saw a clearer separation in β-diversity between the groups based on unweighted UniFrac distances, suggesting that the presence and absence of bacterial OTUs, but not the relative abundance, could contribute more significantly to the observed alterations. This observation is supported by the increased number of observed OTUs in children with ASD, which represents the number of unique OTUs present in the sample. Thus, the number of species but not the relative abundance might be more significantly different between children with ASD and unrelated controls. Previous studies have described increased and decreased α-diversity in children with ASD (Son et al., 2015; Kang et al., 2013; Finegold et al., 2010). Even though richness and diversity are often considered important in resisting invasion of potential pathogenic microbes, it has been hypothesized that increased microbial richness in individuals with ASD could be associated with the presence of more harmful bacteria which could contribute to some symptoms of ASD (Kang et al., 2013). Here, we observed that some bacterial genera that were present in children with ASD were not detected in samples of unaffected controls, such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Sarcina*, *Eubacterium*, *Peptococcus*, *Megasphaera* and Campylobacter, indicating the absence and presence of lower abundance bacteria could potentially be of importance in ASD symptomology and warrants further investigation.

Bacteroidetes and Firmicutes are the two most abundant phyla in the human GI tract (Qin et al., 2010) and disruption of the Firmicutes-to-Bacteroidetes ratio has been implicated in several states of intestinal or systemic inflammation, such as obesity, Type 1 Diabetes and IBS (Ley et al., 2006; Frank et al., 2007; Verdam et al., 2013; Pellegrini et al., 2017). Food

sensitization in children was also associated with lower numbers of Bacteroidetes but higher numbers of Firmicutes (Chen et al., 2016). In ASD, no clear trend in the Firmicutes-to-Bacteroidetes ratio has been described (Strati et al., 2017; Williams et al., 2011; Tomova et al., 2015; Finegold et al., 2010; Kang et al., 2013; El Aidy et al., 2017). Here, an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes in children with ASD was observed. The Firmicutes-to-Bacteroidetes ratio could be linked to two potentially underlying mechanisms in the symptomology of ASD, including the serotonergic signaling system and oxidative stress. First, Firmicutes abundance could be involved in maintaining GI 5-HT homeostasis (El Aidy et al., 2017). A disruption in the serotonergic signaling system in children with ASD has been proposed, elevated levels of whole blood and platelet 5-HT are consistently observed (Gabriele et al., 2014) and GI changes have been suggested to contribute to the hyperserotonemia observed in children with ASD (Anderson et al., 1987; Hanley et al., 1977; Schain & Freedman, 1961). Second, mice exposed to oxidative stress, which has been implicated in the development and pathophysiology of ASD, exhibited a shift to a higher Firmicutes but lower Bacteroidetes abundance (Zhao et al., 2018; Chauhan & Chauhan, 2006; Ashwood et al., 2006*)*. Thus, the Firmicutes-to-Bacteroidetes ratio could have some implications for ASD symptoms, but more research is needed to identify potential mechanisms.

Positive correlations between the bacterial family Coriobacteriaceae and reactive oxygen species were found in infants, suggesting that Coriobacteriaceae could be involved in host inflammatory status (Qasem et al., 2017). Higher levels of Coriobacteriaceae in children with ASD compared to unrelated controls were observed in this and previous studies (De Angelis et al., 2013). Coriobacteriaceae could be involved in host lipid and bile acid metabolism and strong correlations between abundance of Coriobacteriaceae and hepatic triglycerides were identified

(Lahti et al., 2013; Stenman et al., 2013; Claus et al., 2011). An inverse relationship between Coriobacteriaceae and HDL levels as well as an increase in Coriobacteriaceae in response to a Western-style or high-fat diet in rodents has been documented *(*Martínez et al., 2009). Additionally, species of the Coriobacteriaceae family seem to colonize mucosal surfaces and affect epithelial cell metabolism (Clavel et al., 2013). Thereby, Coriobacteriaceae could disrupt the intestinal epithelial barrier and lead to GI barrier dysfunction (Stenman et al., 2013), which has previously been described in children with ASD (de Magistris et al., 2010). Members of the family Coriobacteriaceae have also been implicated in chronic inflammatory conditions and pathologies such as obesity, IBD, colon cancer and bacteremia and might be key microbes that contribute to differences in the microbiota composition between patients with colorectal cancer and healthy controls (Clavel et al., 2013; Chen et al., 2012; Zhang et al., 2009). Interestingly, breast-fed infants had lower levels of Coriobacteriaceae compared to formula-fed infants (Harmsen et al., 2000). Considering the benefits of breastfeeding in development of the microbiota, immune system and the brain, lower levels of Coriobacteriaceae could potentially be advantageous for immune and brain function. In a study investigating the effect of whole grains consumption on immunological improvements in adults, subjects with lower Coriobacteriaceae abundance had greater reductions in the pro-inflammatory cytokine IL-6 after the intervention, indicating that the abundance of that family might predict an individual's immunological response *(*Martínez et al., 2013*)*.

In the present study, higher abundance of *Clostridium* in children with ASD was observed. Additionally, Clostridiales as well as Peptostreptococcaceae and Clostridiaceae, two families within the order of Clostridiales, were also increased in children with ASD. Much interest has been given to the role of the class Clostridia and the genus *Clostridium* in ASD

symptomology (Finegold, 2008). In fact, the first bacterium hypothesized to influence some symptoms of ASD was *Clostridium tetani* in 1998 (Bolte, 1998). Another study two years later described that *Clostridium* species might be of importance in the reoccurrence of ASD symptoms after temporal improvements by vancomycin treatment (Sandler et al., 2000). Since then *Clostridium* clusters have frequently been isolated from feces of children with ASD (Song et al., 2004) and higher levels of these clusters as well as other Clostridial counts in fecal and ileal samples have been described (De Angelis et al., 2013; Grimaldi et al., 2017; Finegold et al., 2010; Finegold et al., 2002; Finegold, 2008; Williams et al., 2011). Likewise, maternal separation and prenatal exposure to valproic acid caused a concurrent increase in the abundance of Clostridiales and deficits in social and repetitive behaviors in mice (El Aidy et al., 2017; de Theije et al., 2014). Diet-induced increases in Clostridiales abundance was also related to poorer cognitive flexibility in mice (Magnusson et al., 2015). Several hypotheses for the involvement of Clostridial species in ASD pathophysiology and symptom development have been proposed, including infection of immunosuppressed, at-risk children with Clostridial spores from the environment and production of enterotoxins and neurotoxins by *Clostridium* species (Finegold, 2008). An increased abundance of Clostridiales in children who developed GI symptoms around the same time of ASD diagnosis could also support the infection hypothesis (Williams et al., 2011). Likewise, Clostridiales could potentially impact the serotonergic signaling system through regulating the transcription of tryptophan hydroxylase 1 (Reigstad et al., 2015; Yano et al., 2015). Transferring GI microbiota that included Clostridiales from non-obese diabetic mice to control mice induced social avoidance behavior and myelination changes in the prefrontal cortex in recipient mice (Gacias et al., 2016). Moreover, metabolic products of some Clostridia species, such as propionate and 3-(3-hydroxxy phenyl)-3-hydroxypropionic acid, have been found in

increased concentration in urine and feces of children with ASD (Keşli et al., 2014; Wang et al., 2012). Surprisingly, bacterial density as assessed by qPCR showed increased numbers of *C. perfringens* in control children (Finegold et al., 2017). However, not all strains within *C. perfringens* could be considered to be harmful bacteria; thus, future studies delineating which strains within *C. perfringens* could potentially contribute to some symptoms of ASD.

In this study, we also observed increases in *SMB53* and *Roseburia*, but decreases in Rikenellaceae, *Butyrivibrio*, *Faecalibacterium*, *Dialister*, and *Bilophila* in children with ASD. Several health benefits have been associated with the bacterial genera *Roseburia*, mostly due to its butyrate-producing abilities that can affect colonic motility, maintain immunity and exert anti-inflammatory properties (Tamanai-Shacoori et al., 2017). Additionally, *Roseburia* abundance was positively correlated with mood in healthy adults (Li et al., 2016) and reduced abundance has been reported in patients with IBS and Ulcerative Colitis (UC) (Shah et al., 2016). However, other studies have also shown that *Roseburia* in obese subjects is positively correlated with Body Mass Index and systemic inflammation (Verdam et al., 2013) and higher levels of *Roseburia* have been observed in children with food sensitization (Chen et al., 2016). *Roseburia* could be also be involved in the development of insulin resistance in mice fed a high-fat, high-sugar diet (Org et al., 2015), suggesting that the physiological effects of *Roseburia* could depend on substrate availability. Rikenellaceae, which was found to be reduced in children with ASD, was previously positively associated with High Intensity Pleasure and activity level in boys (Christian et al., 2015) and was depleted after the onset of colitis in dextran sulfate sodium (DSS) treated mice (Osaka et al., 2017). In line with our results, the abundance of *Dialister* and *Bilophila* was previously shown to be decreased in children with ASD (Finegold et al., 2010; Strati et al., 2017). *Bilophila wadsworthia*, a pathobiont in the human GI tract, is often detected in higher

abundances in patients with inflammatory bowel disorders, suggesting a role of species in this genus in GI health (Devkota & Chang, 2016). Additionally, previous studies have shown that *Bilophila* abundance is susceptible to dietary interventions or prebiotic supplementation in conjunction with improved GI health (Vandeputte et al., 2017; Veiga et al., 2014). Increased abundance of Dialister were linked to decreased levels of the pro-inflammatory cytokine IL-6 after supplementation with whole grains (Martínez et al., 2013).

LEfSe method was used to identify bacterial taxa that are most enriched and could potentially be used as biomarkers among each group (Segata et al., 2011). LEfSe analysis couples standard statistical tests for significance with additional tests for biological consistency and effect relevance to determine OTUs that most likely to explain differences between classes (Segata et al., 2011). Additionally, the effect size provides an estimation of the magnitude observed for each feature. LEfSe analysis confirmed the enrichment of *Clostridium* in the ASD group and Rikenellaceae in the CONT group identified by analysis with generalized linear mixed models. Additionally, enrichment of *Prevotella*, *Collinsella*, and *Megamonas* in children with ASD was identified using LEfSe. The abundance of *Prevotella* could be involved in shaping brain structure and responses, as previous studies have found associations with Novelty Seeking and Reward Dependence as measures of temperament, negative emotional responses, reduced activation of the hippocampus and greater white matter connectivity and hippocampal volume in healthy adults (Kim & Park, 2017; Tillisch et al., 2017). Little is known about the effect of *Megamonas* on the human hosts, but increased *Megamonas* abundance in obese subjects (Chiu et al., 2014) could suggest a role of these genera in inflammatory processes. *Collinsella* has previously been shown to be increased in children with ASD (De Angelis et al., 2013, Inoue et al., 2016; Strati et al., 2017) and higher levels of *Collinsella* were observed in inflammatory

diseases such as colorectal cancer, Type 2 Diabetes Mellitus and food sensitization (Lambeth et al., 2015; Chen 2016).

The mechanisms of the communication between the GI microbiota and ASD symptoms are poorly understood. One study has shown that microbial dysbiosis in an animal model of ASD was associated with alterations in the metabolic pathways that have also been reported in affected children (Lim et al., 2017). Some bacterial metabolites of interest in ASD symptomology are VFAs. VFAs are fermentation products of bacterial carbohydrate (acetate, propionate, butyrate) and protein (isobutyrate, isovalerate, valerate) digestion and several health benefits have been associated with the production of VFAs by the GI microbiota. More than 85% of butyrate produced by the GI microbiota is usually used by colonocytes as an energy source for cell proliferation and differentiation (Bergman, 1990) and is, therefore, important in maintaining the health and integrity of colonic mucosa (Scheppach, 1994). Increased butyrate concentrations have also been proposed to prevent development of colitis (Segain et al., 2000), ameliorate mucosal inflammation, oxidative stress, and strengthen the intestinal epithelium and motility (Canani et al., 2011). However, some adverse effects of VFAs have also been reported, specifically propionate. Propionate can activate the immune response and lead to immune dysfunction (Frye et al., 2017), which might be a strong component in the etiology of ASD (Masi et al., 2017). Furthermore, SCFAs including propionate can induce serotonin production (Reigstad et al., 2015). Hyperserotonemia has been reported in children with ASD and is suggested as a potential metabolic marker for ASD diagnosis (Gabriele et al., 2014). In children with ASD, aberrations in fecal VFA levels with increased propionate concentrations have been reported (Adams et al., 2011a; De Angelis et al., 2013). In the context of our study, acetate, propionate and butyrate concentrations were significantly higher in children with ASD compared

to unaffected controls. It is important to remember that elevated fecal concentrations of these VFAs could be due to increased fermentation of substrates, but could also be explained by decreased absorption or high transit time of food (Schwiertz et al., 2010). Thus, increases in fecal concentrations of VFAs do not necessarily represent the environment in the GI tract. Importantly, some butyrate-producing bacteria such as *Butyrivibrio* and well as the relative abundance of the butyrate-regulating gene BCoAT and the propionate-producing-gene mmDA were decreased in children with ASD. Thus, the increased fecal SCFA concentrations in the children with ASD in this cohort might not be due to increased production, but could mirror a decreased absorption and utilization by colonocytes. In order to provide new insight into the relationship between fecal VFA concentrations and intestinal health, future studies should investigate fecal VFA concentrations in conjunction with makers of intestinal permeability. Additionally, *in vitro* experiments investigating the VFA producing capabilities of the microbiota in children with ASD are warranted.

There is now growing evidence for an association between individual bacteria and some symptoms of ASD. For example, bacterial richness and lower Bacteroidetes-to-Firmicutes ratio were related to ASD symptoms (Kang et al., 2013; Tomova et al., 2015). On the genera level, positive correlations between *Desulfovibrio* abundance and repetitive restrictive behaviors (Tomova et al., 2015) as well as between *Clostridium*, *Prevotella* and *Coprococcus* presence and ASD symptoms (Iovene et al., 2017; Kang et al., 2013) have been described. Here, using regression analysis, we identified that the abundance of Peptostreptococcaceae and *Faecalibacterium* were strong positive predictors of the social deficit scores in children with ASD. Neither of these taxa have previously been associated with symptoms of ASD.

Relative abundance of *Faecalibacterium* can reach up to 15% in some individuals and is one of the most abundant butyrate producing bacteria (Hold et al., 2003; Flint et al., 2012). Therefore, a role of *Faecalibacterium* in health and disease has been suggested. *F. prausnitzii*, to date the only known species within the *Faecalibacterium* genus, is usually regarded as a beneficial bacterium due to its anti-inflammatory properties (Quevrain et al., 2016), improvements of GI barrier function (Carlsson et al., 2013) and support of mucosal immune homeostasis (Hornef & Pabst, 2016). In patients with UC and Chron's diseases lower abundance of *Faecalibacterium* has been observed, potentially due to the production of reactive oxygen species and presence of pathogenic bacteria (Miquel et al., 2013; Swidsinksi et al., 2008). Previously a positive correlation between expression of interferon signaling-associated genes in the blood and abundance of *Faecalibacterium* in the feces of children with ASD was reported, suggesting that abundance of *Faecalibacterium* might be involved in the dysfunction of systemic immunity that is often observed in children with ASD (Inoue et al., 2016). In this study, children with ASD overall had lower levels of *Faecalibacterium*, potentially indicating the presence of an inflammatory state. On the other hand, other studies demonstrated that *Faecalibacterium* could be associated with some disease states, suggesting that potentially the presence of other species within the *Faecalibacterium* genus could be of importance (Swidsinski et al., 2008, Hansen et al., 2012), For example, a correlation of *Faecalibacterium* with obesity and diabetes was observed (Furet et al., 2010) and a subspecies of *F. prausnitzii* might play in a role in onset of atopic dermatitis in infants potentially (Song et al., 2016; Zheng et al., 2016). Likewise, children with food sensitization harbored higher concentrations of *Faecalibacterium* (Chen et al., 2016) and an association with *Faecalibacterium* with negative mood and fatigue interference was described (Li L., et al., 2016; Paulsen et al., 2017).

The second bacterium found to be associated with social deficits scores in children with ASD in this cohort is Peptostreptococcaceae. Peptostreptococcaceae is a family within the order of Clostridiales and encompasses species such as *C. difficile* and other pathogenic clostridia (Milani et al., 2016). Although some studies suggest that Peptostreptococcaceae could contribute to GI homeostasis (Fan et al., 2017), higher abundances in patients with IBD, Ulcerative Colitis, and colorectal cancer were reported (Lavelle et al., 2015; Chen et al., 2012). Furthermore, an overrepresentation of Peptostreptococcaceae in a mice model of colitis and associated with intestinal mucosal ulceration suggests an association between that family and an inflammatory status (Denis et al., 2016; Nagy-Szakal et al., 2013). Lastly, correlations between Peptostreptococcaceae and the right inferior segment of the circular sulcus in patients with IBS, a region for somatosensory and motor function, indicate the potential of this family to impact brain and behavior (Labus et al., 2017).

In conclusion, the results reported herein contribute to the growing knowledge of microbial dysbiosis in children with ASD. We have found similar results to previously described differences in the microbial composition, VFA concentration and GI symptoms between children with ASD and siblings or unrelated controls. Although the GI microbiota in children with ASD in this study harbors some species that are associated with health benefits and is depleted in others which were linked to an inflammatory status, reductions in microbiota that exert a positive effect on the host are also observed. Thus, the role of these individual microbiota within an "ASD microbiome" require further investigation, potentially using animal models. Similar to previous studies, we observe an increased abundance of *Clostridum* in children with ASD. Although we did not find significant associations between *Clostridium* abundance and ASD symptoms, a family within the Clostridiales order, Peptostreptococcaceae positively predicted

social deficit symptoms. Due to strict exclusion criteria, this study had a relatively small sample size, so some results might not offer generalizable information. However, the strict exclusion criteria allowed us to exclude children from the study with recent probiotic, antibiotic or medication use as well as children following any specialty diets or having diagnosed digestive disorders, which are all factors known to influence the microbiota composition. Thus, some of the differences reported herein might be inherent to ASD symptomology. Future studies should investigate whether specific dietary patterns or nutrients are related to differences in the microbiota composition of children with ASD. While this study did not allow us to draw conclusions on causation relationships between the GI microbiota and ASD, these results demonstrate a microbial dysbiosis in children with ASD and that individual bacteria can predict the severity of some symptoms of ASD. Thus, the findings reported here could provide preliminary evidence for future intervention strategies to target specific bacterial strains that are more strongly related to some symptoms of ASD.

4.5 Tables and Figures

Table 4.1 Primer for Real-time quantitative analysis of microbial populations

Target Group	Primer	Sequence (5' – 3')	Annealing Temperature (°C)	Reference
Total Bacteria	Uni331F	TCCTACGGGAGGCAGCAGT	60	Nadkarni
	Uin797R	GGACTACCAGGGTATCTATCCTGTT		et al, 2002
Lactobacillus spp.	LacF	AGCAGTAGGGAATCTTCCA	58	Kok et al,
	LacR	CACCGCTACACATGGAG		1996
Bifidobacterium spp.	Bif164F	GGGTGGTAATGCCGGATG	60	Kok et al,
	Bif662R	CCACCGTTACACCGGGAA		1996
Prevotella spp.	g_prevF	GGTTCTGAGAGGAAGGTCCCC	55	Rinttila et
	g_prevR	TCCTGCACGCTACTTGGCTG		al., 2004
Clostridium	s-Clper-F	GGGGGTTTCAACACCTCC	60	Matsuda
perfringens	ClPER-R	GCAAGGGATGTCAAGTGT		et al.,
				2009
Clostridium difficile	Cdiff_F	TTGAGCGATTTACTTCGGTAAAGA	58	Rinttila T,
	Cdiff_R	CCATCCTGTACTGGCTCACCT		2004
mmdA	mmdAF	AATGACTCGGGIGGIGCIMGNATHCARGA	56	Reichardt
	mmdAR	GATTGTTACYTTIGGIACNGTNGCYTC		et al, 2014
BCoAT	BCoATscrF	GCIGAICATTTCACITGGAAYWSITGGCAYATG	53	Louis and
	BCoATscrR	CCTGCCTTTGCAATRTCIACRAANGC		Flint 2007

Table 4.2 Demographic characteristics of all study participants

Characteristic	ASD (n=26)	CONT (n=32)	p-value
Age (years)	4.1±1.6	4.8±1.8	NS
Gender (n)			
Male	19	19	NS
Female	6	13	
Prefer not to answer	1		
Race/Ethnicity (n (%))			NS
Caucasian	15 (68%)	19 (63%)	
Asian	1 (5%)	5 (17%)	
Black of African American	3 (14%)	3 (10%)	
Hispanic or Latino	1 (5%)	1 (3%)	
Other	2 (9%)	2 (7%)	
Marital Status (n (%))			NS
Married	15 (75%)	25 (83%)	
Single	3 (15%)	1 (3%)	
Divorced	0	1 (3%)	
Cohabitating	2 (10%)	1 (3%)	
Separated	0	2 (7%)	
Parents Level of Education (n (%))			0.001
High School	0	0	
Some College or Technical School	6 (30%)	1 (3%)	
College Graduate	11 (55%)	10 (33%)	
Post-Graduate Work	4 (20%)	19 (63%)	
Annual Income (n (%))			NS
Less than $20,000	1 (5%)	0	
$20,000 to $60,000	4 (20%)	11 (37%)	
$60,000 to $80,000	5 (25%)	7 (23%)	
$80,000 to $100,000	2 (10%)	5 (17%)	
More than $100,000	5 (25%)	8 (27%)	
Prefer not to answer	3 (15%)	0	
Health Care Coverage (n (%))			NS
Private paid by employer	7 (35%)	6 (20%)	
Private paid by employer and household	10 (50%)	16 (53%)	
Medicaid, Medicare	3 (15%)	8 (27%)	
Combination	1 (5%)	0	
Weight (kg)	20.5±7.8	21±0.7	NS
Height (meters)	1.07±0.1	1.10±0.15	0.08
Mean BMI and Percentile (BMI-for-age)			
Male	16.8 (70^{th})	16.3 (75^{th})	NS
Female	15.1 (50^{th})	16.8 (82^{nd})	

Table 4.2 (cont.)

Characteristic	ASD (n=26)	CONT (n=32)	p-value
Nutritional Supplement use (n (%))			
Yes	12 (46%)	14 (44%)	NS
No	14 (54%)	18 (56%)	
Route of Birth (n (%))			
Vaginal	11 (42%)	18 (56%)	NS
Planned C-section	6 (23%)	4 (13%)	
Emergency C-section	9 (35%)	10 (31%)	
Gestational age (n (%))			NS
<37 weeks	3 (12%)	2 (6%)	
37-42 weeks	19 (73%)	27 (84%)	
>42 weeks	4 (15%)	3 (9%)	
Early Feeding Mode (n (%))			0.03
Breast-fed only	5 (20%)	17 (53%)	
Breast-fed in combination with formula	17 (65%)	13 (41%)	
Formula only	4 (15%)	2 (6%)	
Antibiotics use in early life (n (%))			NS
Yes			
No	4 (15%)	2 (6%)	
	22 (85%)	30 (94%)	
Picky Eater			0.1
Yes	13 (50%)	10 (31 %)	
No	13 (50%)	22 (69%)	
More than 20 foods in diet (n (%))			0.0007
Yes	12 (46%)	29 (90%)	
No	14 (54%)	3 (10%)	
Repetitive eating pattern			NS
Yes	14 (54%)	13 (36%)	
No	12 (46%)	19 (64%)	

Data expressed as mean±SD or n

Table 4.3 Characteristics of children with ASD

Characteristics	Mean ± SD	Comments
Average SOCDEF T score[1]	52±9	(Level 4 – moderate)
Average age to see changes in behavior (years)	1.44±0.69	
Average age of diagnosis (years)	2.4±1.3	
Receiving behavior therapy (n(%))		Most Common: ABA, Speech therapy, physical therapy, occupational therapy, music therapy, social integration
Yes	18 (69%)	
No	8 (31%)	
Sensory problems (n)		
Yes	17 (65%)	
No	9 (35%)	
CAM usage (n)		Most common: Chiropractic, Mind-Body Medicine, Nutrition Supplements
Yes	14 (54%)	
No	12 (46%)	

Data expressed as mean±SD or n

[1]Social deficit score measured by PDDBI-SV; range of T-scores: <22-30 Level 1 (unlikely); 31-35 Level 2 (borderline); 36-40 Level 3(mild); 41-59 Level 4 (moderate); 60-69 Level 5 (severe); 70->80 Level 6 (extreme)

CAM, Complementary and Alternative Medicine

ABA, Applied behavior Analysis

Table 4.4 Differences in GI Severity scores and stool consistency between children with ASD (ASD) and unaffected controls (CONT).

Characteristic	**ASD (n=26)**	**CONT (n=32)**	**p-value**
GI Severity Score[1]	2 (0-3)	0 (0-1)	0.005
Constipation	1 (0-2)	0 (0-1)	NS
Diarrhea	0	0	NS
Stool Smell	0 (0-1)	0	0.006
Flatulence	0 (0-1)	0	0.04
Abdominal Pain	0 (0-1)	0	0.07
Stool Consistency (n)[2]			0.05
Type 1 (separate hard lumps)	0	1	
Type 2 (sausage shaped but lumpy)	5	3	
Type 3 (sausage-shaped with cracks on surface)	5	13	
Type 4 (smooth and soft)	12	15	
Type 5 (soft blobs)	2	0	
Type 6 (mushy)	2	0	
Type 7 (watery)	0	0	

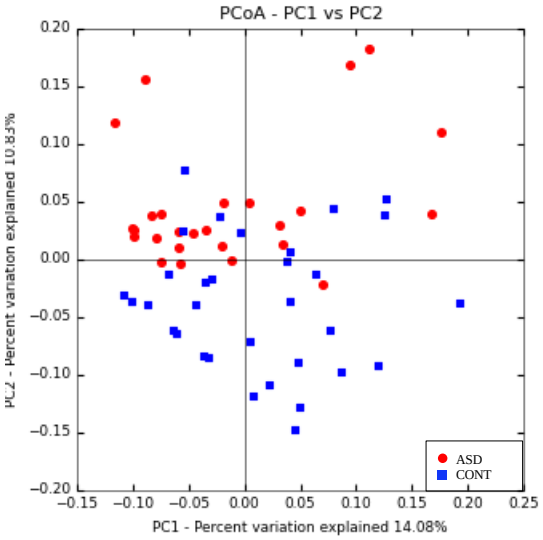
Data expressed as median (IQR) or n

[1]GI severity scores were derived from the GI severity index (possible range 0-7)

[2]Stool consistency was measured using the Bristol Stool chart

Figure 4.1 Principal co-ordinate analysis based on unweighted UniFrac Distance (a) and weighted UniFrac Distance (b) generated from fecal samples of children with ASD (ASD) and unrelated controls (CONT).

a)



b)

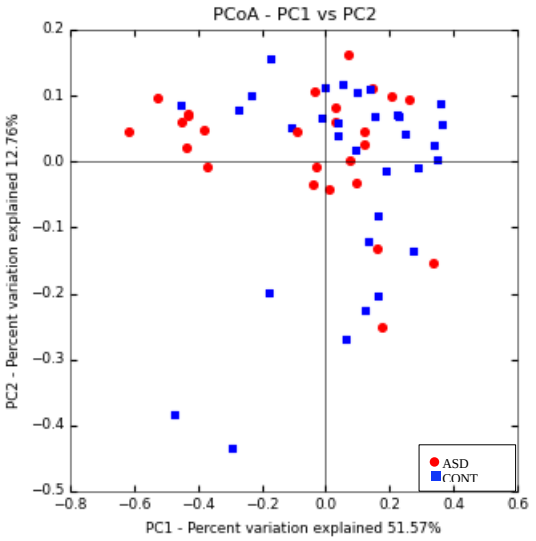


Table 4.5 α-Diversity measures between children with ASD (ASD) and unaffected controls (CONT).

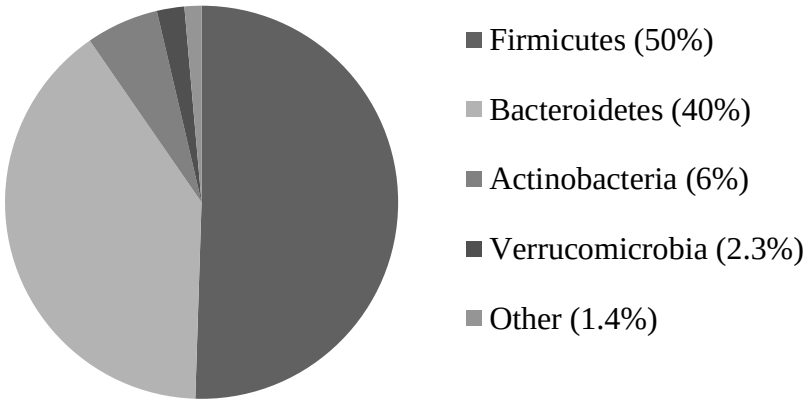
Diversity/Richness Measure	ASD (n=26)	CONT (n=32)	p-value
Shannon Index	5.5 (5.1-5.7)	5.4 (4.9-5.7)	NS
Simpson Index	0.95 (0.91-0.96)	0.94 (0.92-0.95)	NS
Observed OTUs	580 (539-607)	553 (495-580)	0.08
Chao 1 Index	527 (475-562)	500 (442-536)	NS

Data expressed as median (IQR)

ASD, Autism spectrum Disorder group; CONT, unrelated controls group; IQR, interquartile range

Figure 4.2 Pie graphs depicted the abundance of bacterial phyla in feces of in children with ASD (a) and unaffected controls (b).

a)



b)

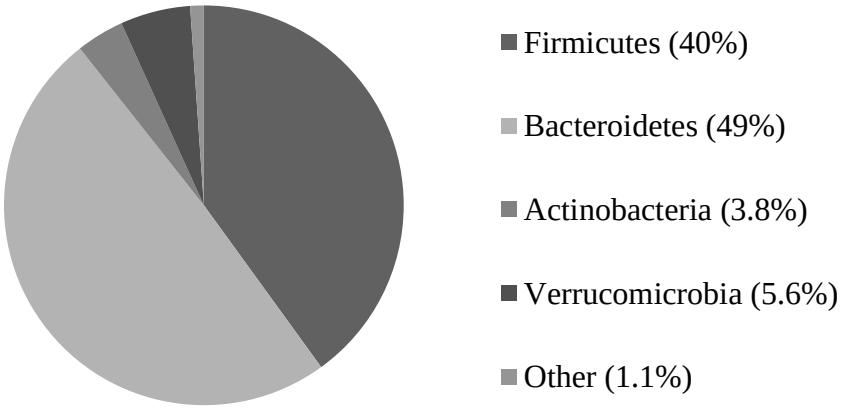


Table 4.6

a) Relative abundances of bacterial taxa detected in feces of children with ASD (ASD) and unaffected controls (CONT).

Phyla	**ASD (n=26)**	**CONT (n=32)**	**p-value**
Euryarchaeota	0.0009 (0.0006-0.003)	0 (0-0.001)	NS
Actinobacteria	3.1 (1-9.8)	7.62 (1.05-16.73)	NS
Bacteroidetes	46.7 (16.7-55.5)	53.4 (42.3-63.2)	0.07
Cyanobacteria	0 (0-0.002)	0.002 (0-0.01)	NS
Firmicutes	45.8 (37.8-66.2)	36.5 (30.5-48.7)	0.03
Fusobacteria	0 (0-0)	0 (0-0)	NS
Proteobacteria	0.47 (0.13-1.25)	0.65 (0.25-1.26)	NS
Tenericutes	0 (0-0)	0 (0-0)	NS
Verrucomicrobia	0.17 (0.02-2.65)	0.4 (0.01-7.65)	NS

Order	**ASD (n=26)**	**CONT (n=32)**	**p-value**
Bacteroidales	0 (0-0)	0 (0-0)	NS
Streptophyta	0.008 (0.003-0.02)	0.002 (0-0.01)	NS
Clostridiales	2.99 (2.11-6.41)	2.23 (1.4-3.36)	0.07
RF32	0.09 (0.01-0.13)	0 (0-0.002)	NS
RF39	0 (0-0)	0 (0-0)	NS

Family	**ASD (n=26)**	**CONT (n=32)**	**p-value**
Coriobacteriaceae	0.04 (0.007-0.21)	0.01 (0.002-0.03)	0.04
RF16	0 (0-0)	0 (0-0)	NS
Rikenellaceae	0.53 (0.05-3.56)	2.94 (0.98-6.58)	0.005
S24-7	0 (0-0)	0 (0-0)	NS
Barnesiellaceae	0.02 (0.006-0.79)	0.03 (0.006-1.82)	NS
Christensenellaceae	0.002 (0-0.05)	0.002 (0-0.02)	NS
Clostridiaceae	0.29 (0.14-1.5)	0.17 (0.08-0.44)	0.07
EtOH8	0 (0-0)	0 (0-0)	NS
Lachnospiraceae	7.1 (5.6-11.1)	6.03 (3.8-9.2)	NS
Peptostreptococcaceae	0.04 (0.01-0.07)	0.02 (0.009-0.03)	0.05
Mogibacteriaceae	0.01 (0-0.04)	0.01 (0.002-0.03)	NS
Erysipelotrichaceae	0.34 (0.16-1.02)	0.26 (0.13-1.02)	NS
Enterobacteriaceae	0.02 (0.002-0.23)	0.02 (0.005-0.09)	NS

Genera	**ASD (n=26)**	**CONT (n=32)**	**p-value**
Archea – Euryarchaeota			
Methanobrevibacter	0.0009 (0.0006-0.003)	0 (0-0.001)	NS
Actinobacteria			
Bifidobacterium	1.88 (0.56-4.52)	1.54 (0.58-3.00)	NS
Adlercreutzia	0.05 (0.009-0.21)	0.04 (0-0.19)	NS
Collinsella	0.26 (0.009-1.68)	0.04 (0.004-0.09)	NS
Eggerthella	0.05 (0.02-0.09)	0.03 (0.006-0.11)	NS

Table 4.6 (cont.)

Genera	ASD (n=26)	CONT (n=32)	p-value
Slackia	0 (0-0.001)	0 (0-0.002)	NS
Bacteroidetes			
Bacteroides	29.4 (13.3-46.7)	37.97 (51.6-55.3)	NS
Parabacteroides	0.88 (0.19-3.29)	1.46 (0.27-3.25)	NS
Prevotella	0.02 (0.01-0.10)	0.009 (0.006-0.02)	NS
Alistipes	0.0003 (0-0.002)	0.001 (0-0.009)	NS
Butyricimonas	0 (0-0.0005)	0 (0-0)	NS
Odoribacter	0.009 (0.0007-0.15)	0.06 (0.003-0.37)	NS
Paraprevotella	0 (0-0.0007)	0 (0-0.001)	NS
Firmicutes			
Staphylococcus	0.0008 (0.003-0.02)	0.007 (0.003-0.01)	NS
Enterococcus	0 (0-0.001)	0 (0-0.001)	NS
Lactobacillus	0.0007 (0-0.002)	0 (0-0.001)	NS
Leuconostoc	0 (0-0)	0 (0-0)	NS
Lactococcus	0.0009 (0-0.007)	0.002 (0-0.009)	NS
Streptococcus	0.11 (0.07-0.49)	0.15 (0.05-0.35)	NS
Turicibacter	0.02 (0.003-0.16)	0.02 (0.002-0.04)	NS
02d06	0.002 (0.0007-0.01)	0.001 (0-0.003)	NS
Clostridaceae_ Clostridium	0.33 (0.05-0.66)	0.07 (0.02-0.22)	0.02
SMB53	1.16 (0.55-3.39)	0.56 (0.34-1.48)	0.07
Sarcina	0.0008 (0.0006-0.02)	0 (0-0)	NS
Eubacterium	0.07 (0.01-0.13)	0.03 (0.02-0.06)	NS
Anaerostipes	0.04 (0.01-0.08)	0.05 (0.006-0.09)	NS
Blautia	1.34 (0.65-2.72)	0.85 (0.56-1.59)	NS
Butyrivibrio	0.008 (0.001-0.02)	0.02 (0.005-0.09)	0.003
Coprococcus	3.73 (1.99-4.75)	2.64 (1.49-3.75)	NS
Dorea	0.78 (0.57-1.39)	0.71 (0.38-1.07)	NS
Lachnospira	0.15 (0.02-0.61)	0.38 (0.09-0.63)	NS
Roseburia	0.46 (0.27-0.75)	0.29 (0.08-0.51)	0.01
Peptococcus	0 (0-0)	0 (0-0)	NS
Anaerotruncus	0.01 (0.003-0.02)	0.006 (0.002-0.02)	NS
Peptostreptococcaceae_Clostridium	0.0007 (0-0.002)	0 (0-0.003)	NS
Faecalibacterium	6.59 (3.47-11.96)	10.59 (7.04-16.17)	0.05
Oscillospira	0.41 (0.29-0.55)	0.43 (0.25-0.66)	NS
Ruminococcus	1.99 (0.67-3.08)	1.58 (0.57-3.79)	NS
Acidaminococcus	0 (0-0)	0 (0-0.0005)	NS
Dialister	0.12 (0.008-0.72)	0.64 (0.32-1.63)	0.005
Megamonas	0.001 (0.0007-0.003)	0 (0-0.001)	NS
Megasphaera	0 (0-0.0006)	0 (0-0)	NS
Phascolarctobacterium	0.006 (0-0.18)	0.0007 (0-0.003)	NS
Succiniclasticum	0 (0-0)	0 (0-0)	NS

Table 4.6 (cont.)

Genera	ASD (n=26)	CONT (n=32)	p-value
Veillonella	0.006 (0.002-0.03)	0.02 (0.005-0.05)	NS
Catenibacterium	0 (0-0)	0 (0-0)	NS
Coprobacillus	0 (0-0.03)	0 (0-0.006)	NS
Holdemania	0.006 (0-0.01)	0.007 (0.003-0.02)	NS
Fusobacteria			
Fusobacterium	0 (0-0)	0 (0-0)	NS
Proteobacteria			
Sutterella	0.09 (0.02-0.37)	0.28 (0.08-1.14)	NS
Bilophila	0.003 (0.006-0.02)	0.006 (0-0.08)	0.04
Campylobacter	0 (0-0)	0 (0-0)	NS
Haemophilus	0.003 (0.001-0.05)	0.01 (0.002-0.08)	NS
Verrucomicrobia			
Akkermansia	0.17 (0.02-2.65)	0.41 (0.01-7.65)	NS

Data expressed as median (IQR)
ASD, Autism Spectrum Disorder; CONT, unaffected controls; IQR, interquartile range

Figure 4.3 LDA score histogram from LEfSe analysis depicting enrichment of most abundant (LDA score >3) bacterial taxa in ASD (red) and CONT (green) children

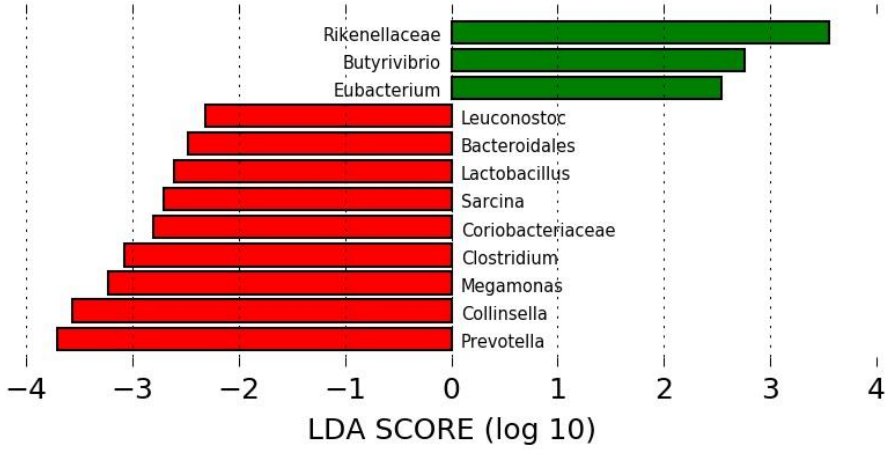
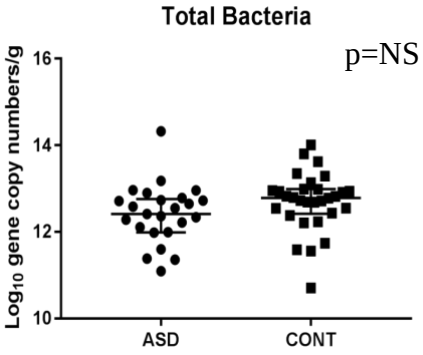
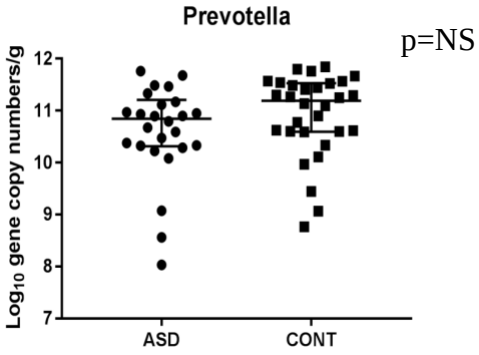


Figure 4.4 Bacterial densities in feces of ASD and CONT children measured by qPCR

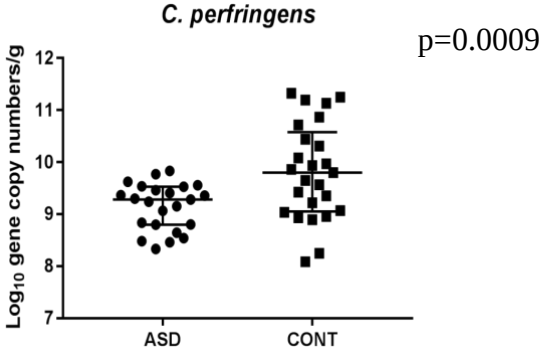
a)



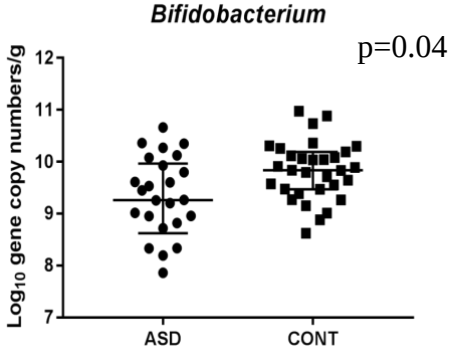
b)



c)



d)



e)

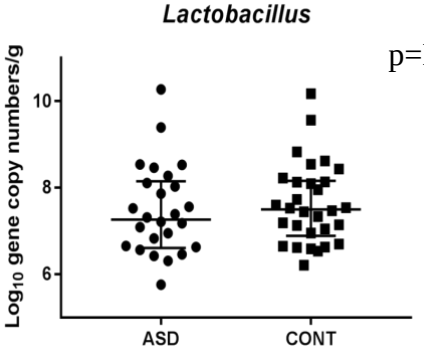
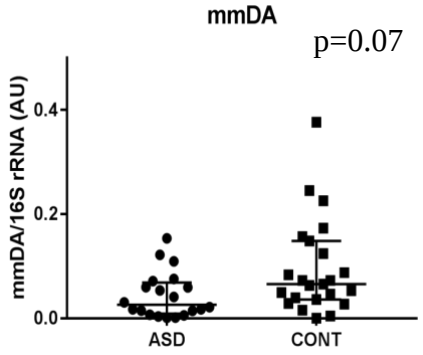
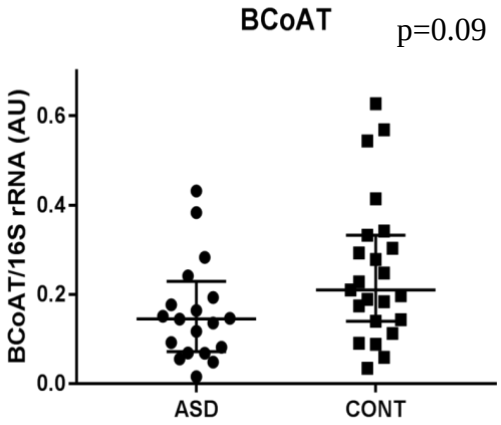


Figure 4.4 (cont.)

f)

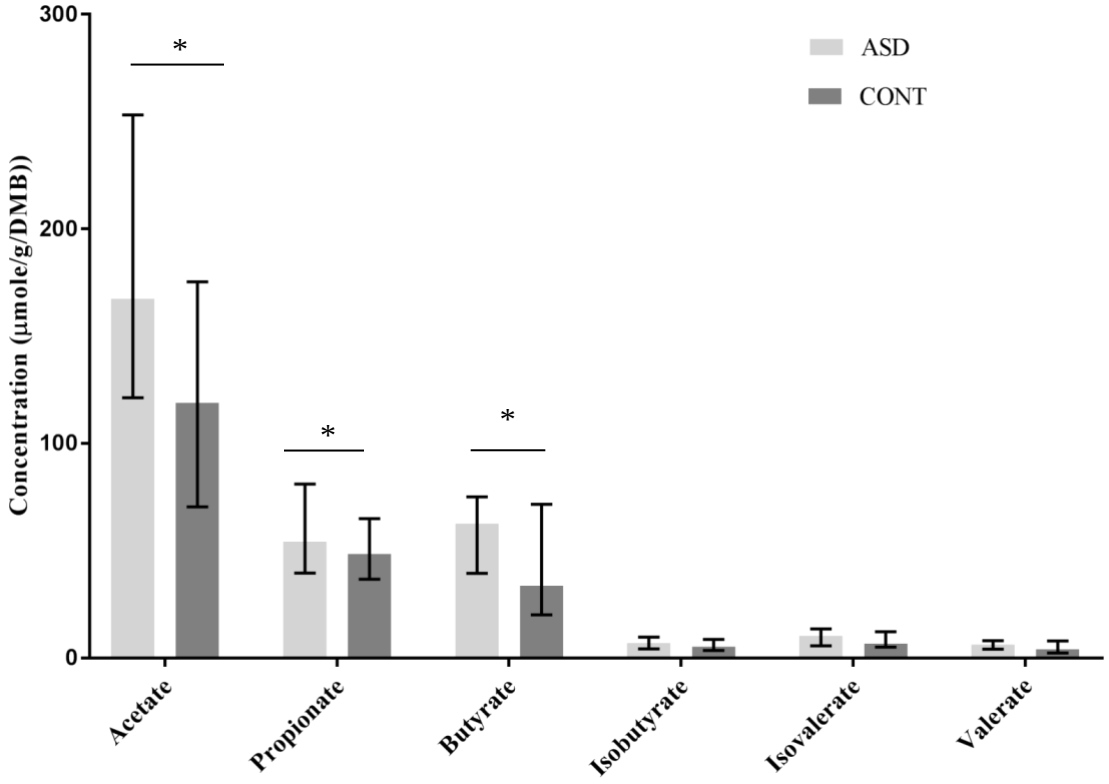


g)



Data is expressed as Median (IQR)
ASD, Autism Spectrum Disorder; CONT, unaffected controls; AU, arbitrary unit; IQR, interquartile range

Figure 4.5 Differences in VFA concentrations between CONT and ASD



Median (IQR) *p≤0.05
CONT, unaffected controls; ASD, Autism Spectrum Disorder; DMB, dry matter basis; IQR, interquartile range

Table 4.7 Regression analysis model showing fecal bacterial taxa predicting social deficit scores.

Variable	Parameter Estimate B	Standardized Estimate β	Standard Error	t-Value	P-value	Squared Semi-partial Corr Type II
Intercept	43.5		3.02	14.41	<.0001	.
Faecalibacterium	0.9	0.63	0.27	3.39	0.003	0.35
Peptostreptococcaceae	14.1	0.33	7.96	1.77	0.09	0.09

Bacteria expressed as relative abundance derived from 16S rRNA sequencing. SOCDEF scores: social deficit scores derived from PDDBI-SV.

Table 4.8 Bacterial abundance and GI symptoms/stool consistency

a) Correlation between Bacteria and GI symptom severity in children with ASD

Bacterial Taxa	**ρ**	**p-value**
Bacteroidetes	-0.46	0.02
Cyanobacteria	-0.43	0.03
Firmicutes	0.44	0.02
Streptophyta	-0.43	0.03
Bacteroidales	0.40	0.04
Rikenellaceae	0.44	0.02
Clostridiaceae	0.41	0.04
Methanobrevibacter	0.39	0.05
Bacteroides	-0.43	0.03
Butyricimonas	0.45	0.02
Enterococcus	0.38	0.05
Holdemania	0.41	0.04

b) Difference in *Holdemania* abundance based on stool consistency in children with ASD

Stool Type	***Holdemania* abundance**	**p-value**
Soft	0.005 (0.0004-0.01)	0.01
Normal	0.003 (0-0.01)	
Hard	0.007 (0.002-0.02)	

c) Correlation between Bacteria and GI symptom severity in unaffected control children

Bacterial Taxa	**ρ**	**p-value**
Streptococcus	-0.39	0.03
Lachnospiraceae	-0.39	0.03
Coprococcus	-0.45	0.01
Blautia	-0.34	0.06
Bilophila	0.32	0.08

d) Difference in bacterial abundance based on stool consistency in unaffected control children

Bacterial taxa	**Soft**	**Normal**	**Hard**	**p-value**
Streptophyta	0 (0-0)	0.005 (0.001-0.03)	0 (0-0.004)	0.05
Alistipes	0 (0-0)	0 (0-0.002)	0.004 (0.002-0.03)	0.05
Dialister	3.15 (3.15-3.15)	0.87 (0.58-1.81)	0.32 (0.005-0.53)	0.01

Data expressed as median (IQR)

stool type was assessed using the Bristol stool chart; hard: type 1-3, normal: type 4, soft: type 5-7; GI severity score was assessed using GI severity index

IQR, interquartile range

CHAPTER 5

Nutrition, GI Microbiota and Microbial Metabolites in Children with ASD[4]

Abstract

Background/Aims: Diet is considered one of the most influential environmental factors in determining the composition of the gastrointestinal (GI) microbiota. Increasing reports describe a microbial dysbiosis in children with Autism Spectrum Disorder (ASD) and the impact of some bacterial taxa on some symptoms of ASD has been recognized. Likewise, children with ASD are often described as picky eaters and decreased intakes of fiber-rich foods, including fruits and vegetables, have been reported. Differences in nutrient intakes between children with ASD and unaffected controls are often observed. However, the impact of diet on the microbiota in children with ASD has not been explored.

Methods: Herein, differences in diet between children with ASD and unrelated controls as well as the influence of feeding behavior, habitual dietary patterns and short-term nutrient intakes on the microbiota composition in children with ASD was investigated. Additionally, dietary moderators of the association between microbiota abundance and social deficit score were analyzed. Fecal samples, 3-day diet records and a food frequency questionnaire (FFQ) were collected from children with ASD (n=26) and unrelated controls (n=32) aged 2-7 years. Social deficit scores were assessed using the Pervasive Developmental Disorder Behavior Inventory-Screening Version (PDDBI-SV). Bacterial composition was assessed by 16S rRNA sequencing and quantitative PCR.

Results: Lower intakes of insoluble fiber, pectin, vitamin C, and dairy, but higher intakes of snacks and sweets were observed in children with ASD. Feeding behaviors (i.e., picky eating,

[4] Berding K, Donovan SM. Diet and Feeding Behavior are Related to Microbiota Composition in Children with Autism Spectrum Disorder. *Front Neurosci*. 2018. *Under Review*

repetitive eating behavior, diet variety) were associated with unique microbial profiles and VFA concentrations. For example, higher abundances of Clostridiales were higher in children with ASD and repetitive eating patterns and reduced diet variety. Dietary patterns (DP) were empirically derived the FFQ. In children with ASD two DPs were identified. DP1, characterized by intakes of vegetables, starchy vegetables, legumes, nuts and seeds, fruit, grains, juice and dairy, was associated with lower abundance of Enterobacteriaceae, *Lactococcus*, *Roseburia*, *Leuconostoc*, and *Ruminococcus*. DP2, characterized by intakes of fried foods, Kid's meals, condiments, protein foods, snacks and starchy foods was associated with higher abundance of Barnesiellaceae, *Alistipes*, and lower abundance of *Streptophyta* as well as higher concentrations of propionate, butyrate, isobutyrate, valerate, and isovalerate. Moderation analysis revealed no significant moderation effect of diet on the microbiota-symptom interaction in ASD and social deficit socres were not associated with diet-induced microbial profiles. However, some GI symptoms were related to microbiota profiles linked to feeding behaviors and dietary patterns.

Conclusion: These results document that nutrient intake and dietary patterns affect the GI microbiota and GI symptoms in children with ASD. However, additional research is required to determine whether diet can be a modifiable moderator of how the microbiome affects ASD symptoms.

5.1 Introduction

The gastrointestinal (GI) microbiota is influenced by a variety of environmental factors, including geographical region, presence of pets in the household and dietary factors. It has been estimated that more than 50% of microbial changes can be attributed to diet (Zhang et al., 2010). Studies in humans and other mammals have shown that the GI microbiota composition can be

clustered based on diet (herbivore, omnivore or carnivore) (Muegge et al., 2011). Short-term changes of dietary intake over a 5-day period have shown to impact the composition and function of the human GI microbiota, while habitual dietary patterns are thought to be more notable associated with long-term stability of the gut microbiota (David et al., 2014; Wu et al., 2011). In one study, a short-term (10 days) dietary intervention did not lead to a change the bacterial enterotype that was associated with an individual's long-term dietary patterns (Wu et al., 2011). Additionally, we have previously demonstrated that habitual dietary patterns are associated with a distinct microbial profile and microbial stability over a 6 months period in children 4-8 years of age (Berding et al., 2017).

Aberrations in the GI microbiota in children with Autism Spectrum Disorder (ASD) have been reported and associations between specific microbial genera and some symptoms of ASD were described (Tomova et al., 2015). Likewise, differences in nutrient intake and dietary patterns between children with ASD and unaffected controls are commonly observed. Achieving adequate nutrition intake often presents a challenge in children with ASD and some nutrient deficiencies have been identified (Ledford & Gast, 2006; Liu X, et al., 2016). Picky eating, food selectivity and food refusal are common behaviors observed in children with ASD and some children might eat as little as five foods (Cermak et al., 2010). Picky eating behaviors might be a manifestation of repetitive behavior patterns, ritualistic or externalizing behaviors (Johnson et al., 2014). Others suggest that picky eating might be a reflection of the child's resistance to change, inflexibility, sensory sensitivities, inadvertent reinforcement of negative mealtime behaviors, GI problems and oral motor delay (Johnson et al., 2014; Cermak et al., 2010).

Diet-induced changes in microbiota composition can lead to increased risk of developing certain diseases (e.g., inflammatory bowel diseases), whereas a healthier long-term dietary

pattern may be more beneficial in promoting a microbial profile that could potentially protect against diseases (Albenberg & Wu, 2014). An interrelationship between dietary intake, neurodevelopment, and cognitive function has been demonstrated in healthy children and most results are attributed to the direct effect of dietary components on the central nervous system (Khan et al., 2015; Khan et al., 2015a). However, the role of microbial changes induced by dietary modification in influencing cognitive processes is not well described. In malnourished individuals it has been hypothesized that the microbiome is causally related to neurological abnormalities (Goyal et al., 2015). A few animal models have shown that diet-induced changes in the GI microbiota could contribute to observed behavioral changes (Li et al., 2009; Pyndt Jørgensen et al., 2014). For example, rodent fed a meat-containing diet had an increase microbial diversity as well as improved working and reference memory (Li et al., 2009). Other studies confirmed the effect of high calorie diets on brain function with co-occurring changes in the GI microbiota composition (Magnusson et al., 2015). A Western-style diet, which is characterized by high fat intake, negatively affected anxiety-like behavior and memory capabilities and was associated with an increased ratio of Firmicutes-to-Bacteroides as well as an increase in *Proteobacteria* and *Spirochaetes* (Ohland et al., 2013).

Knowing the impact of diet on the microbiota composition as well as the often limited nutritional intake in children with ASD, it is surprising that previous research analyzing the microbiota in this population is insufficient in informing about the effect of diet on the microbiota composition. To fill this gap, the goal of this study was to systematically investigate the impact of dietary patterns and nutrient intake on the GI microbiota in children with ASD and test whether specific dietary factors could moderate the relationship between the GI microbiota and some symptoms of ASD. We hypothesized that children with ASD with a dietary pattern

high in fruit, vegetables and grains will harbor a more beneficial microbiota compared to a children with a dietary pattern high in animal and processed foods. We further hypothesized that healthy food groups will have a more favorable moderating effect on the relationship between specific bacterial taxa and symptoms of ASD compared to unhealthy food options.

5.2 Materials and Methods

Study Design, Participants, Fecal Sample Collection and Analysis, 3-Day Food Diary, and Assessment of ASD Symptoms

The study design, participant recruitment and sample collection are described in Chapter 3. Fecal samples for microbiota and VFA analysis were collected and analyzed as referred to in Chapter 3. Likewise, nutrient intake measured by 3-day food records and ASD symptom information were collected as described in Chapter 3.

Food Frequency Questionnaire

A revised version of the semi-quantitative Youth and Adolescent Food Frequency Questionnaire (YAQ) was completed by the parents to estimate their child's usual food and beverage intake over the past year (Bandini et al., 2010). The questionnaire was modified for parent report as previously described and has been used in the ASD population to assess food selectivity (Bandini et al., 2010). The revised YAQ contained 156 food items, compared with the original 169. For example, food items such as coffee or alcoholic drinks were excluded from the questionnaire due to the age of the participants. The original YAQ has been tested for reproducibility and validity (Rockett et al., 1997). Parents were asked to estimate the frequency with which their children consumed a specified portion size of each of the foods listed over the

preceding year. The questionnaire had five possible responses ranging from "never or less than once per month" to "more than 4 times per week". The 156 foods items were grouped a priori into 13 food groups (e.g. fruits, vegetables, grains, sweets) (**Appendix B**) based on general dietary guidelines for children and using methods similar to those described in previous studies of dietary patterns and disease (Evans et al., 2012). To estimate the number of servings of any food group, each response was converted to the corresponding frequency factor (Harvard T.H Chan School of Public Health Nutrition Department's Food Group Serving Table) and summed over all the food items to get the average servings of a specific food group per day.

Statistics

All data was analyzed using SAS 9.4 (SAS Institute, Cary, NC). All data was expressed as mean ± SD. Level of significance was set at $p<0.05$.

Differences between groups in nutrient and food group intake were analyzed using mixed models. Model fit was assessed using the chi-square-to-df ratio. Values <2 were indicate an appropriate model fit. Relationships between nutrient and food group intake and microbiota composition were initially analyzed using Spearman correlation.

Dietary patterns data derived from the YAQ were analyzed using Principal Component Analysis and Factor Analysis with Varimax rotation. Retention of the number of factors in the final analysis was based on Eigenvalues ≥ 2, percentage of variability explained, interpretability of factors and Scree plots. Food groups with a factor loading >0.35 were considered significant contributors to each overall dietary pattern. Factor scores were calculated for each participant by summing the reported frequency of intake for each food in a food group standardized by the factor loadings. Scores for dietary patterns were calculated for each participant and stratified as

either falling above or below the median. Differences in microbiota composition, VFA concentrations and nutrient intake between participants falling above or below the median for each dietary pattern were analyzed using mixed models.

Hierarchical multiple regression was performed to determine whether food groups or nutrients can moderate the relationship between the GI microbiota and ASD symptoms. Dietary variables was denoted as the moderator variable, bacterial genera was denoted as the independent variable and social deficit scores were denoted as the dependent variable. In order to avoid multicollinearity variables were mean centered by subtracting the sample mean from each participant's score. Using stepwise regression, dietary factors and microbiota abundance were identified that significantly predict SOCDEF symptoms. Entry and stay levels were 0.05 and 0.1, respectively, for independent variables to stay in the model. For each model identified through stepwise regression model adequacy was tested. Variance Inflation Factor (VIF) was calculated to determine whether multicollinearity is present in the model. Variables with a VIF of greater than 2 were removed from the model. Additionally, correlations between the independent variables were tested. Normality of the residuals was measured using Shapiro-Wilk. The SPEC test was used to test homogeneity of Variances and the Durbin-Watson test assessed residual correlation. Lastly, Cook's D was calculated for each model to determine the influence of individual observations. The Mallow's Cp value was calculated and plots were reviewed to assess the goodness of fit for each model. Following the identification and model adequacy testing of significant independent variables, moderation analysis was performed using the Process macro available in SAS (Hayes, 2012). Age, gender, season, height, weight and BMI were included as co-variates in the model. A moderating effect occurs when R^2 significantly increases after adding the interaction term and the interaction term shows a significant effect. A

complete moderation occurs when the predictor and moderator variable are not significant after the interaction term was added to the model. If the predictor and moderator variable remain significant, then a partial moderation occurred with significant main effects. Significant interactions were probed using the simple slope analysis and followed-up with the Johnson-Neyman (J-N) technique (Bauer & Curran 2005; Hayes & Matthes, 2009). Simple slopes (i.e., standardized β) analysis estimates the conditional effect of the independent variable (microbiota) when the moderator (diet) is equal to the mean as well as plus and minus one standard deviation from the mean (Hayes, 2012). The J-N technique is then applied to identify regions of the moderator where the effect of the independent on the dependent variable is significant. This technique can be applied when the moderator is a continuum and avoids arbitrary selection high, medium and low levels of the moderator.

5.3 Results

Nutrient Intake

Nutrient intake derived from the 3-day food record of both groups is shown in **Table 5.1**. Overall, there was no difference in macronutrient intake between the ASD and CONT group. Total dietary fiber intake did not differ between the groups; however, children with ASD tended to have a lower intake of insoluble dietary fiber ($p=0.09$), but higher intake of pectin ($p=0.09$) compared to CONT. With regard to vitamins and minerals, the only difference was observed in intake of vitamin C, with the ASD group having lower ($p=0.01$) intakes compared to CONT children.

Intake of Servings per Day of Food Groups Differs between Groups

Besides differences in the intake of nutrients, some differences in the intake of servings per day of specific food groups derived from the YAQ were observed (**Table 5.2**). Children with ASD tended to eat fewer servings per day of dairy (p=0.05), but more servings per day of snacks (p=0.09) and sweets (p=0.1) compared to CONT children.

Relationship between Microbiota or VFA and Food Groups or Nutrient Intake

In order to investigate whether different food groups or nutrients are associated differently with microbiota composition among the two groups, we used Spearman correlation to analyze potential relationships. Several correlations for each group between nutrient and food group intake and microbiota abundance and VFA concentrations were observed (**Supplemental Table 5.1 and 5.2**).

Associations between Picky Eating Behavior and Repetitive Eating Pattern and Microbiota Composition in Children with ASD

In order to analyze potential differences in the gut microbiota composition based in eating behavior of children with ASD, we investigated the microbiota composition and VFA concentration between children with ASD based on parent report of picky eating and repetitive eating patterns. Surprisingly, no differences in SOCDEF and total GI severity scores based on picky eating behavior or repetitive eating pattern were found. However, we found differences in the nutrient and food group intake as well as microbiota composition based on picky eating behavior and repetitive eating patterns (**Table 5.3**).

Picky Eating Behavior: Children with ASD described as picky eaters had higher abdominal pain scores compared to non-picky eaters. In regard to nutrition, children described as

picky eaters had lower intakes of total fat, monounsaturated fatty acids, and protein foods, but higher intakes of juice. Regarding the microbiota composition, children with picky eating behavior had higher abundance of Coriobacteriaceae and EtOH8. On the genera level, children described as picky eaters had higher abundance of *Ruminococcus* and *Holdemania*, but lower relative abundance of *Bacteroides* and *Phascolarctobacterium*. Lastly, higher concentrations of isobutyrate and isovalerate were observed in children with ASD described as picky eaters.

Including 20 Foods in Diet: Children with ASD that included less than 20 foods in their diet had a higher BMI compared to children with ASD who included more than 20 foods in their diet. Likewise, higher scores of total GI severity, flatulence and abdominal pain were observed in children including less than 20 foods in their diet. In regard to their nutritional intake, children eating 20 foods or less had lower intakes of pectin, vitamin C, niacin, vitamin B6, folate and selenium, but higher intakes of added sugars. Regarding the microbiota composition, children with 20 foods or less in the diet had higher levels of Actinobacteria, Coriobacteriaceae, Clostridiales, *Bifidobacterium*, *Collinsella*, *Lactobacillus*, and *Acidaminococcus*, but lower abundances of Bacteroidetes, Cyanobacteria, *Eggerthella*, *Bacteroides*, *Dialister* and *Anaerotuncus*. Concentrations of valerate tended to be higher in children with ASD who include less than 20 foods in their diet.

Repetitive Eating Pattern: Children with ASD and repetitive eating patterns had a higher BMI. Nutritionally, children with repetitive eating patterns had lower intake of pectin, vitamin C, potassium, and copper. On the bacterial order level, lower abundance of Streptophyta but higher abundances of Clostridiales were observed in children with repetitive eating patterns. Additionally, children with ASD and repetitive eating patterns had higher abundance of Coriobacteriacea and Actinobacteria, but lower abundance of Verrucomicrobia and

Cyanobacteria. Lastly, on the genus level children with repetitive eating behaviors had higher levels of *Collinsella* and *Butyrivibrio*, but lower abundance of *Adlercreutzia*, *Eggerthella*, *Dialister*, *Coprobacillus* and *Akkermansia*.

Dietary Patterns, Participant Characteristics, Microbiota Composition and VFA Concentration in ASD Group

Dietary Patterns in both groups: Using factor analysis, two distinct dietary patterns for the both groups were identified. In children with ASD, Dietary Pattern 1 (DP1-ASD) was characterized by an intake of vegetables, legumes, nuts and seeds, fruit, starchy vegetables, grains, juice and dairy. Dietary Pattern 2 (DP2-ASD) was characterized by an intake of fried foods, Kid's meals, condiments, protein foods, snacks and starchy foods. Refined carbohydrates were present in both dietary patterns but were more associated with DP1-ASD. Fish, sweets and sweetened beverages were not significantly associated with either dietary pattern.

In the CONT group, one Dietary Pattern (DP1-CONT) was characterized by intakes of sweets, Kid's meals, fried foods, snacks, starchy foods, dairy and sweetened beverages, whereas Dietary Pattern (DP2-CONT) was characterized by intakes of fish, vegetables, protein foods, fruit, and juice. Refined carbohydrates contributed significantly to both dietary patterns, whereas grains, starchy vegetables, condiments and legumes, nuts and seeds did not contribute significantly to either dietary pattern. The factor loading matrix can be found in **Table 5.4**.

Dietary patterns, nutrient intake and microbiota composition in ASD group: For the ASD group, participant were dichotomized by category of factor score (above or below the median) in order to analyze differences in participant characteristics, nutrient intakes, VFA concentration

and bacterial abundance based on long-term dietary pattern (**Tables 5.5 – 5.7**). No differences in participant characteristics (e.g., age, gender etc.) based on dietary patterns were observed.

In DP1-ASD, children above the median had higher intakes of fruit, vegetables, legumes, nuts and seeds, refined carbohydrates and starchy vegetables, but lower intakes of sweets compared to children falling below the median. Additionally, children above the median in DP1-ASD had higher intake of folate, vitamin E, vitamin A and insoluble dietary fiber but lower intakes of vitamin B_{12}. Additionally, we children above the median in DP1 lower abundance of Enterobacteriaceae, *Lactococcus*, *Roseburia*, *Leuconostoc* and *Ruminococcus* compared to children below the median. No significant differences in VFA concentrations at baseline between factor score categories in DP1-ASD were observed.

In DP2-ASD, children above the median had lower intakes of vegetables, legumes, nuts and starchy vegetables. Furthermore, children above the median in DP2-ASD had higher intakes of vitamin B_{12} as well as total and refined grains, but lower intake of niacin and vitamin B_6 compared to children below the median in DP2-ASD. Additionally, children above the median in DP2-ASD had higher abundance Barnesiellaceae and *Alistipes* and lower abundance of *Streptophyta*. Higher levels of propionate, isobutyrate, valerate and isovalerate were observed in children above the median in DP2-ASD.

Dietary patterns, nutrient intake and microbiota composition in CONT group: For the CONT group, participant characteristics, VFA concentration and bacterial abundance dichotomized as falling above or below the median based on dietary patterns are shown in **Tables 5.8-5.10**. In DP1-CONT children above the median tended to have a higher BMI

compared to children below the median. There was no difference in participant characteristics in either dietary pattern.

In DP1-CONT, children above the median consumed higher amounts of refined carbohydrates, sweets, snacks, dairy foods, and Kid's meals as well as, but lower intake of vitamin B_{12}, zinc, and soluble dietary fiber. Higher abundances of *Eubacterium* as well as higher concentrations of valerate were observed in children above the median in DP1-CONT.

In DP 2-CONT, children above the median had higher intakes of fruit, starchy vegetables, protein foods, snacks, fish, and grain food as well as higher amounts of total fat, saturated fatty acids, omega-3 fatty acids, and sodium, but lower intakes of refined grains. The microbial profile in children above the mean in DP2-CONT was characterized by higher abundance of Cyanobacteria, Rikenellaceae, Streptophyta, *Enterobacteriaceae* and *Coprobacillus*, and lower abundance of Verrucomicrobia and *Akkermansia*.

Moderation Analysis

Moderation analysis was performed to determine whether dietary intake influences the interaction between the GI microbiota and ASD symptoms using hierarchical multiple regression. In Step 1, using stepwise regression, Lactobacillus, Peptostreptococcaceae, *Dialister* and *Faecalibacterium* were determined to significantly predict SOCDEF scores (**Table 5.11a**). In Step 2, using regression analysis calcium and total grain intake were identified as significantly predictors of SOCDEF scores (**Table 5.11b**). Additionally, eating behavior measures were tested for predicting SOCDEF scores. Neither repetitive eating patterns nor including twenty foods in the diet or picky eating behavior were significant predictors of SOCDEF scores.

There was no significant interaction between the nutrient intake variables and bacterial abundance in predicting SOCDEF symptoms. Thus, no moderation effect of dietary components on the relationship between bacterial taxa and SOCDEF scores occurred.

5.4 Discussion

Differences in the GI microbiota composition between children with ASD and unaffected controls are increasingly described and are suggested to influence some symptoms of ASD. Likewise, picky eating and inadequate nutrient intake are commonly reported in children with ASD (Cermak et al., 2010). Even though diet represents a major environmental factor for influencing the GI microbiota composition, studies investigating the microbiota in children with ASD lack in systematically describing the diet-microbiota interaction. Therefore, we collected information on dietary habits (food frequency questionnaire, 3-day food record) as well as fecal samples from children with ASD and unaffected controls to investigate how nutrient intake and dietary patterns impact the GI microbiota composition. Additionally, specific nutrients or food groups were analyzed for their potential to moderate the microbiota-brain connection in ASD. To the best of our knowledge, this is the first study reporting an association between dietary intake and microbiota composition in children with ASD.

Achieving adequate nutritional intake presents a big challenge in children with ASD due to GI symptoms, food allergies, metabolic abnormalities or problematic eating behaviors. Approximately 90% of children with ASD experience some sort of feeding-related concern which often present as multidimensional problems (Ledford & Gast, 2006; Nadon et al., 2011). Here, we report lower intake of dairy foods, insoluble fiber, pectin and vitamin C, but higher intakes of snacks and sweets in children with ASD compared to unaffected controls. Several

studies have found that children with ASD have a strong preference for starches, snack and processed foods, while rejecting fruits, vegetables or protein (Bicer & Alsaffar, 2013; Al-Farsi et al., 2011; Mahli et al., 2017). In contrast to reports outlining that the total mean average of nutrient deficiencies is higher in children with ASD (Zimmer et al., 2012; Shmaya et al., 2015), participants in both groups in this study met the Dietary Reference Intakes for 4-to-8-year old children for most vitamins and minerals except for potassium, vitamin D, vitamin E, and choline. Furthermore, children with ASD did not meet the RDA for vitamin K intake and consumption of fewer foods from the dairy food group were observed in children with ASD (Shearer et al., 1982; Schreck et al., 2004; Herndon et al., 2008). One explanation for the lower intake of dairy foods in children with ASD in this study could be the current trend for using the Gluten-free/Casein-free (GFCF) diet in the ASD population. The interest in the GFCF diet stems for the theory that children with ASD have a limited ability to break down gluten and casein. Undigested gluten and casein can then translocate into the periphery, bind to opioid receptors in the Central Nervous System and impact neuronal development, cognitive function, attention, learning and behavioral symptoms associated with ASD (Whiteley et al., 1999). Even though clinical studies using GFCF diets for ameliorating some symptoms of ASD have not provided conclusive evidence for its efficacy (Knivsberg et al., 2002; Whiteley et al., 2010; Elder et al., 2006), the GFCF diet is still widely used in the ASD community. Thus, even though children following a specialty diet (including GFCF diet) were excluded from the study, parents of children with ASD could still have offered less dairy foods to their children. Although low intake of dietary fiber in children with ASD has been reported (Bicer & Alsaffar, 2013), here only the intake of insoluble dietary fiber and pectin, but not total dietary fiber differed significantly between the groups. This might be attributable to the fact that children in both groups only consumed about 65% of the

recommended amounts of fiber. Additionally, children in both groups consumed only about half of the recommended 3 servings per day of vegetables and only about 20% of the total grains were consumed as whole grains, which are both high-fiber food sources. This observation is in line with reports that children in the United States often consume inadequate amounts of dietary fiber and fail to meet dietary guidelines for fiber-rich foods, such as fruit, vegetables and whole grains (McGill & Devareddy, 2015; Frazier-Wood et al., 2016). Although dietary intake differed between children with ASD and unaffected controls in this cohort, the differences did not reach the extent as often described in the literature.

Previously published studies lack in systematically investigating the effect of dietary habits of children with ASD on the composition of the GI microbiota. Of the studies analyzing the GI microbiota composition in children with ASD, only Son et al. collected dietary information and analyzed the macronutrient intake of study participants (Son et al., 2015). Other studies only collected information on specialty diets (Horvath et al., 1999; Finegold et al., 2002; Parracho et al., 2005; Kang et al., 2013; Wang et al., 2011) or probiotic and supplement use (Wang et al., 2011; Adams et al., 2011a; Kang et al., 2013). Kang et al. concluded that dietary intake of children with ASD was not significantly correlated with bacterial abundance (Kang et al., 2013); however, the researchers only assessed dietary intake by asking whether the child was following a GFCF diet, used probiotics or nutrition supplements or consumed seafood. In order to investigate the impact of nutrient and food intake on the microbiota and microbial products, we first performed simple correlation analysis to determine whether dietary intake correlates with individual bacterial taxa and VFA concentrations. Not surprisingly, we found correlations between intake of specific nutrients and the abundance of microbiota and VFA concentrations in both children with ASD and unaffected controls. It is commonly known that the GI microbiota

and bacterial metabolites are associated with nutrient intake and dietary patterns. Some bacteria commonly affected by dietary intake are *Bifidobacterium* spp, *Lactobacillus* spp, *Bacteroides* spp, *Alistipes*, *Bilophila*, *Clostridium*, *Roseburia*, *Eubacaterium*, *Enterococcus*, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, *Escherichia coli*, *Helicobacter pylori*, and *Streptococcus* spp (Singh et al., 2017). Thereby, different food groups and different macronutrients can have distinct effects on the microbiota composition and microbial metabolites. For example, dairy intake was negatively associated with species richness and diversity, whereas vegetable intake increased *Lachnospira* abundance and fruit intake decreased Firmicutes-to-Bacteroidetes ratio and *Ruminococcus gnavus* abundance (Smith-Brown et al., 2016). Irregardless of the protein source, general protein consumption correlates with overall bacterial diversity. Bacterial genera such as *Clostridium*, *Bacteroides* and *Lactobacillus* contain proteases that can break down undigested protein (Rawlings et al., 2013). Increased animal protein intake corresponded to higher abundances of Bacteroides and *Alistipes* (De Filippo et al., 2010) and a high-beef diet resulted in higher counts of *Bacteroides* and Clostridia in human subjects (Singh et al., 2017). Additionally, a diet high in protein but low in carbohydrate reduced the concentration of *Roseburia, Eubacterium rectale* and butyrate (Russell et al., 2011). Likewise, these alterations in specific bacterial taxa associated with protein consumption can be linked to changes in the host physiology (Singh et al., 2017). The amount and type of fat both can also shape the composition and diversity of the GI microbiota. For example, a high-fat diet causes a decrease in the abundance of lactic acid bacteria, but an increase Clostridiales and *Bacteroides*, whereas a low-fat diet was associated with increases in concentrations of *Bifidobacterium* (Singh et al., 2017). Mice fed lard, a source of saturated fat, had an increase in *Bacteroides*, *Turicibacter* and *Bilophila*, whereas an increase in beneficial bacteria such as

Bifidobacterium, *Lactobacillus*, *Streptococcus* and *A. muciniphila* was observed in animals fed fish oil, a source high in unsaturated fats (Caesar et al., 2015). Carbohydrate is the most widely studied macronutrient regarding the ability to impact the GI microbiota. Indigestible carbohydrates, such as dietary fiber, are fermented by the GI microbiota and have the potential of modulating its composition. A diet higher in dietary fiber can increase bacterial abundance and richness and beneficial bacteria such as *Lactobacillus*, *Bifidobacteria* and *Roseburia*, but decrease harmful bacteria such as Clostridia (Singh et al., 2017). Associations between macronutrient intake and bacterial abundance previously reported are in line with correlations observed herein. For example, daily intake of servings of protein positively correlated with *Bacteroides* abundance in children with ASD, whereas total protein intake positively correlated with *Alistipes* and Clostridiales in control children. Furthermore, total fat consumption was positively correlated with *Clostridium* in control children. In children with ASD, insoluble fiber negatively correlated with Clostridiales abundance and pectin consumption was negatively correlated with Clostridiaceae abundance. Interestingly, in children with ASD abundance of *Faecalibacterium* positively correlated with unhealthy food group (fried food), but negatively correlated with a beneficial food (fruit). Previous research has shown that a healthy dietary pattern, the Mediterranean diet, increased the abundance of *F. prausnitzii* (Haro et al., 2016). These results provide first evidence that nutrient and food group intake influence the microbiota composition and VFA concentration in children with ASD and suggest that dietary intake should be considered when analyzing microbial composition in this population.

Next, we investigated whether picky eating behavior or repetitive eating patterns were associated with a distinct dietary intake and microbiota composition in children with ASD. Picky eating behaviors and repetitive eating pattern are commonly observed in children with ASD and

potentially pose another point of influence on the microbiota composition (Provost et al., 2010; Diolordi et al., 2014). In this study population, about half of the children with ASD were perceived by their parents as being picky eaters or having a repetitive eating pattern. Picky eating behavior is characterized by strong food preferences and rejecting new foods (Taylor et al., 2015). Assessment of picky eating ranges from a single item questions to more complex questionnaires (Taylor et al., 2015). Some single item questions include "Is your child a picky eater" which was utilized to assess picky eating in this study (Mascola et al., 2010). Research has consistently demonstrated that picky eaters have less variety in their diet compared to non-picky eaters (Taylor et al., 2015). Because diet variety has previously been measured by including 20 food groups in the diet (Quick et al., 2014), we also measured diet variety by asking parents whether the child's diet includes 20 or more foods. Limited data investigating the differences in nutrient intake between picky eaters and non-picky eaters among children with ASD is available. One study showed that children with ASD described as selective eaters ate fewer total food, but information on nutrient intake was not available (Tanner et al., 2015). Likewise, children with ASD and severe food selectivity did not meet the recommended amounts of some nutrients and children with restricted diets had poor protein intake and deficiencies in essential amino acids (Arnold et al., 2003). Children with ASD following a restricted diet also had worse Healthy Eating Index scores and the intake of some vitamins, minerals and grains was lower compared to children with ASD who were non-selective eaters (Graf-Myles et al., 2013). In children without ASD, it is commonly observed that picky eaters have reduced intakes of fruit and vegetables, whole grain products, fish and other seafood, but higher intakes of meat, savory snacks and confectionery compared to their peers (Taylor et al., 2015). Additionally, intakes of energy, vitamin E, folate and dietary fiber have been reported to be lower in picky eaters (Taylor et al.,

2015). Interestingly, one study investigating the nutrient intake between picky eaters among children with ASD and unaffected controls found no differences between the groups (Lockner et al., 2008), suggesting that picky eating behavior but not necessarily ASD status can impact nutrient intake.

Herein, among children with ASD those described by their parents as being picky eaters had lower intakes of total fat, monounsaturated fatty acids, and protein foods, but higher intakes of juice. Generally, consuming fewer calories from fat is regarded a healthier dietary pattern; however, the type of fat consumed is important when considering the effects on the body. For example, saturated fats can increase inflammation, metabolic disease and the risk of developing coronary heart disease and stroke (Kennedy et al., 2009; Nettleton et al., 2017), whereas unsaturated fats exert anti-inflammatory properties and promote a lean and metabolically healthy phenotype (Buckley & Howe, 2009; Lunn & Theobald, 2006). Some of the differences in the microbiota composition observed could be attributed to the differences in nutrient intake. Among children with ASD those with picky eating behavior had higher abundance of Coriobacteriaceae, EtOH8, *Ruminococcus* and *Holdemania*, but lower relative abundance of *Bacteroides* and *Phascolarctobacterium* as well as higher concentrations of isobutyrate and isovalerate. Some of these abundances of bacteria have been described in the literature as being associated with host physiology, suggesting that the microbiota of children with ASD who are picky eaters could be less beneficial. Higher abundance of *Bacteroides* has shown to support development of immune tolerance and avoidance of allergy and asthma (Wexler, 2007) and some mucolytic *Ruminococcus* species (e.g., *R. gnavus* and *R. torgues*) have been observed to be increased in patients with Chron's Disease and Ulcerative Colitis (Png et al., 2010). Abundance of *Holdemania* correlated with clinical indicators of impaired lipid and glucose metabolism

(Lippert et al., 2017), while higher *Phascolarctobacterium* abundance can have beneficial effects on the host by producing VFA (Louis et al., 2010) and is correlated with positive mood (Li et al., 2016). Lastly, higher concentrations of isobutyrate and isovalerate could suggest microbial metabolic changes and increased dietary energy extraction form the microbiota in picky eaters among children with ASD. Whether isobutyrate is beneficial or harmful remains to be determined (Jost et al., 2014). In states of low butyrate concentrations, colonocytes can metabolize isobutyrate (Jaskiewicz et al., 1996) and the microbiota of patients with IBS produced 25% more branched chain fatty acids compared to healthy individuals (Van Nuenen et al., 2004).

Similar to observations based on picky eating behaviors, differences in nutrient intake and microbiota composition among children with ASD and limited diet variety were detected. Interestingly, children with ASD consuming less than 20 foods in their diet had a higher BMI and GI severity scores compared to children eating more than 20 foods. Regarding nutritional intake, children with ASD who are consuming less than 20 foods in their diet had lower intakes of pectin, vitamin C, niacin, vitamin B_6, folate and selenium, but higher intake of added sugars. Pectin is a soluble fiber that can be fermented by the GI microbiota to yield SCFA. Pectin has been postulated to maintain the balance of the GI microbiota (Licht et al., 2010) and can have a significant effect at the individual species level (Chung et al., 2016). For example, pectin utilization is widespread among the *Bacteroides* genus (Dongowski et al., 2000), which was decreased in children with ASD including less than 20 foods in their diet. Higher abundance of Actinobacteria as well as *Bifidobacterium*, which also belongs to the phylum Actinobacteria, were detected in children with ASD including less than 20 foods in their diet. Actinobacteria abundance was found to be higher in children with Attention Deficit/Hyperactivity Disorder

(Aarts et al., 2017) and was associated with brain structure and function in humans (Fernandez Real et al., 2015). *Bifidobacterium* is regarded as a beneficial microbe due to its ability to improve epithelial barrier function, to reduce allergic symptoms, to prevent pathogen infections, and to produce bioactive metabolites such as SCFA, vitamins or polyunsaturated fatty acids which contribute to intestinal function and immune modulation (Hidalgo-Cantarana et al., 2017; Bottacini et al., 2014). However, higher abundance of *Bifidobacterium* could also indicate a less mature microbiota composition since *Bifidobacterium* is shown to decrease with age (Ottman et al., 2012). Furthermore, abundance of *Bifidobacterium* could be involved in the dopaminergic signaling pathway, thus potentially impacting brain activity and behavior (Aarts et al., 2017). Another genus, *Lactobacillus,* that is associated with health benefits, such as decreased inflammation, protection against pathogen, manipulating of host's immune response, vitamin synthesis, improvement of nutrient absorption and enhanced barrier function (Patten & Laws, 2015; Madsen et al., 1999), was increased in children with ASD who had less than 20 foods in their diet. Due to the benefits from the bacteria itself as well as their metabolic products (Patten & Laws, 2015), *Lactobacillus* and *Bifidobacterium* are among the most frequently used strains in probiotic products (Otles et al., 2003). Despite the increased abundance of some beneficial bacteria in children with ASD who consume less than 20 foods in their diet, other bacterial taxa have been implicated to have negative effects on host health. Lower abundance of Bacteroidetes has been noted in children with food sensitization (Chen et al., 2016) as well as in mice exposed to oxidative stress (Zhao et al., 2018). Importantly, oxidative stress was also postulated to be involved in the development of ASD symptoms (Chauhan & Chauhan, 2006; Ashwood & Wakefiled, 2006). A lower proportion of Bacteroidetes as well as *Bacteroides*, as observed in children with ASD and less than 20 foods in their diet, may also be related to obesity (Ley et al.,

2005; Johnson et al., 2017; Haro et al., 2016). Intriguingly, some health outcome measures (i.e., BMI) were higher in children who have less than 20 foods in their diet. The abundance of another potentially pathogenic bacteria, Clostridiales, was also higher in children with ASD and less than 20 foods in their diet. The abundances of Clostridiaceae and *Clostridium* are often associated with ASD and species within the Clostrdiaceae family could potentially affect ASD symptomology by producing entero- and neurotoxins (Finegold, 2008). On the genus level, higher abundance of *Collinsella* was observed which has also been related to inflammatory diseases such as colorectal cancer (Chen et al., 2012), Type 2 Diabetes Mellitus (Lambeth et al., 2015) and food sensitization (Chen et al., 2016). Higher levels of *Acidaminococcus* could potential be detrimental to intestinal function (Gough et al., 2015) and enterocyte proliferation due to its ability to use glutamate as a sole energy source. Glutamate is an important amino acid for intestinal barrier function, amino acid metabolism and nitrogen balance (Rogosa, 1969). Likewise, the presence of *Dialister* was a significant predictor for the treatment response in children with IBS (Shaw et al., 2016). Lastly, *Anaerotruncus* in conjunction with other genera has been found to attenuate inflammation by stimulating regulatory T-cells in a mouse model of colitis (Atarashi et al., 2013; Joosens et al., 2011).

Repetitive eating patterns, defined as the preference for eating the same foods, resulted in differences in nutrient intake and microbiota composition. Similar to children consuming less than 20 foods in their diet, children with repetitive eating patterns had a higher BMI and GI severity scores and consumed lower amounts of pectin, vitamin C, potassium, and copper. The microbial profile associated with repetitive eating patterns were characterized by lower abundance of Streptophyta, Verrucomicrobia, Cyanobacteria, *Adlercreutzia*, *Eggerthella*, *Dialister*, *Coprobacillus* and *Akkermansia*, but higher abundances of Actinobacteria,

Clostridiales, Coriobacteriacea, *Collinsella* and *Butyrivibrio*. Despite the higher abundance of beneficial bacteria such as Butyrivibrio, children with ASD and a repetitive eating pattern had higher GI severity scores. This observation could partly be explained by the abundances of some bacteria that have been linked to potential negative consequences for the host such as Clostridiales or *Collinsella*. The abundance of Coriobacteriaceae was associated with an increased inflammatory status (Qasem et al., 2017), increased pro-inflammatory cytokines, altered activities of antioxidant enzymes (Chauhan & Chauhan, 2006) and inflammatory diseases such as IBD or colon cancer (Clavel et al., 2013). Additionally, species within the Coriobacteriaceae family could potentially disrupt the epithelial barrier and lead to GI dysfunction by colonizing mucosal surfaces (Stenman et al., 2013; Turnbaugh, 2012). *Akkermansia* is a mucin-degrading bacterium that has been suggested to be a marker for GI health (Png et al., 2010; Swidsinski et al., 2011), due to the high abundance in healthy mucosa, the involvement in inducing immune response pathways, chemotaxis, cell adhesion and maturation of B- and T-cells and influencing the expression of genes involved in metabolic and signaling pathways (Derrien et al., 2011). Lastly, a decreased abundance *Adlercreutzia* was found in children with IBS (Shaw et al., 2016).

Third, we investigated the effect of long-term dietary patterns on the microbiota composition in children with ASD. Habitual long term dietary patterns have been suggested to influence the GI microbiota more dramatically than short term nutrient changes and might be associated with long-term stability (Lozupone et al., 2012; David et al., 2014; Wu et al., 2011). Microbiota composition can be clustered based on dietary habits (Muegge et al., 2011) and healthier long-term dietary patterns, i.e., increased consumption of fruits, vegetables and whole grains and less intake of processed foods, simple carbohydrates and fried foods are associated

with a microbial profile that could potentially protect against diseases (Albenberg & Wu, 2014). We have previously shown that a dietary pattern characterized by intake of fish, protein foods, refined carbohydrates, vegetables, fruit, juice and sweetened beverages, kid's meals and snacks and sweets, was associated with higher relative abundance of Bacteroidetes, *Bacteroides* and *Ruminococcus* and lower abundance of *Bifidobacterium*, *Prevotella*, *Blautia* and *Roseburia.* A dietary pattern characterized by intake of grains, dairy and legumes, nuts and seeds, was associated with higher relative abundance of Cyanobacteria and *Phascolarctobacterium* and lower abundance of *Dorea* and *Eubacterium* (Berding et al., 2018). Diets such as the Western-style diet, Gluten-free diets, vegan, vegetarian or Mediterranean diets can influence the microbiota composition. In healthy individuals, following a Gluten-free diet caused a decrease in *Bifidobacterium*, *C. lotuseburense*, *F. prausnitzii* and *Lactobacillus* and an increase in pathogenic bacteria including Enterobacteriaceae and *E. coli* (De Palma et al., 2009; Singh et al., 2017). Mediterranean diet, which is rich in dietary fiber and antioxidants, can increase total bacteria, *Bifidobacterium*, *Lactobacillus*, *Prevotella* and *Roseburia* and fecal SCFA (Singh et al., 2017; De Filippis et al., 2015). Defining long-term dietary pattern associated with a microbial profile that could have beneficial effects on some symptoms of ASD could provide new guidelines for future longer term treatment strategies. Here we described two dietary patterns in children with ASD that are linked to a distinct microbiota composition.

In a healthier dietary pattern DP1-ASD, children above the median had higher intakes of fruit and vegetables, legumes, nuts and seeds, refined carbohydrates and starchy vegetables, but lower intakes of sweets compared to children falling below the median. Additionally, children above the median in DP1-ASD had higher intake of folate, vitamin E, vitamin A and insoluble dietary fiber but lower intakes of vitamin B_{12}. Children falling above the median in DP1-ASD

also had lower abundance of Enterobacteriaceae, *Lactococcus*, *Roseburia*, *Leuconostoc* and *Ruminococcus* compared to children below the median. These abundances could be indicative of a less beneficial microbial profile. For example, in children with ASD, Enterobacteriaceae levels was highest in ASD group compared to unaffected controls and children with Pervasive Developmental Disorder not otherwise specified (De Angelis et al., 2013). Higher abundance of Enterobacteriaceae was proposed as marker of microbiota dysbiosis and epithelial dysfunction (Shin et al., 2015) and increased abundance of members of the Enterobacteriaceae family is often observed in patients with Chron's Disease and Ulcerative Colitis (Frank et al., 2007). Furthermore, the abundance of Enterobacteriaceae in mice fed a high-fat diet was correlated with an increase in endotoxin concentrations (Kim et al., 2012). Albeit the often positive impacts of *Lactococcus*, *Leuconostoc* and *Roseburia* on the host physiology, in some conditions there bacteria could exert negative consequences for the host. Some reports of *Leuconostoc* bacteremia (Casanova-Roman et al., 2003; Ishiyama et al., 2011) might denote species within this genus as opportunistic pathogens and pathogenic properties of *Leuconostoc* species in the pediatric population who are already immunocompromised have been reported (Scano et al., 1999; Florescu et al., 2008). Additionally, *Leuconostoc* might be implicated in causing some human disease (Barreau & Wagener, 1990) and two species, *L. mesenteroides* and *L. carnosum* were higher in patients with Celiac disease (Sanz et al., 2007). *Roseburia*, known for its health benefits elicited by the production of butyrate (Tamanai-Shacoori et al., 2017), was shown to be positively correlated with BMI and systemic inflammation in obese subjects (Verdam et al., 2013) and could be involved in the development of insulin resistance in mice fed a high-fat high-sugar diet (Org et al., 2015). These observations suggest the physiological effects of *Roseburia* and other beneficial microbes could depend on the substrate availability and presence of other

microbes. Considering the relatively healthier eating behavior, mirrored by higher intakes of fruit, vegetables, legumes nuts and seeds and insoluble fiber in children above the median in DP1-ASD, the lower abundance of *Lactococcus*, *Leuconostoc* and *Roseburia* in children above the median in DP1-ASD might not be of physiological importance.

In an unhealthier dietary pattern DP2-ASD, children that fell above the median had lower intakes of vegetables, legumes, nuts and starchy vegetables and had higher intakes of total and refined grains compared to children below the median in DP2-ASD. This unhealthier eating pattern was associated with a microbiota composition that harbored more less beneficial microbes. Barnesiellaceae, which was increased in children above the median in DP2-ASD, was associated with sedentary lifestyles and predicted by the percentage of body fat (Bressa et al., 2017). Furthermore, the abundance of *Alistipes* might be associated with increased abdominal pain, GI inflammation, systemic infections and major depressive disorder (Naseribafrouei et al., 2014; Jiang 2015; Moschen et al., 2016; Boente et al., 2000). Interestingly, children above the median had higher levels of propionate, and the branched chain fatty acids isobutyrate, valerate and isovalerate. Increased VFAs have been reported in children with ASD (Wang et al., 2012). The exact mechanism is unknown, but some studies suggest that VFAs could play a role in ASD pathophysiology (Thomas et al., 2012; MacFabe, 2011; Foley et al., 2015). Even though children above the median in DP2 displayed an unhealthier eating pattern and a less beneficial microbiota, no differences were observed in measures of host physiology, such as GI symptoms or social deficit scores.

Contrary to our hypothesis, no associations between diet-induced microbial profile and social deficit scores were observed. A direct correlation between nutrients (e.g., vitamin A or folate) and severity of ASD symptoms has been reported (Liu X, et al., 2016). A healthy,

nutrient-rich diet has been proven to support good mental health (O'Neil et al., 2013; Khan et al., 2015). Due to the profound effect of diet on the GI microbiota composition and the newly acquired knowledge about the gut-brain-connection, the GI microbiota was proposed to be key mediator in the diet-brain health connection potentially through direct interaction of the GI microbiota and its metabolic products with enteric neurons (Hanstock et al., 2004; Furness et al., 1999; Dawson et al., 2016). A decrease in microbial diversity caused by consumption of highly processed foods was linked to increased risk for mental disorder (Dawson et al., 2016). In an animal model, supplementation with lean-beef caused marked diet-induced shifts in the GI microbiota diversity as well as improved reference and working memory and decreased anxiety-like behavior (Li et al., 2009). Likewise, high calorie or high fat diets impacted brain function with co-occurring changes in the GI microbiota composition (Magnusson et al., 2015; Bruce-Keller et al., 2015). Interestingly, one study found that feeding a high-fat or high-sucrose diet to rodents resulted in increased percentages of Clostridiales and Bacteroidales, two bacteria orders that have been found to be altered in children with ASD. Behaviorally, these mice also displayed poorer cognitive flexibility compare to mice fed a normal chow diet (Magnusson et al., 2015). A Western-style diet, which is characterized by high fat intake, negatively affected anxiety-like behavior and memory capabilities and was associated with an increased ratio of Firmicutes to Bacteroides as well as an increase in *Proteobacteria* and *Spirochaetes* (Ohland et al., 2013). Lastly, rodents treated with a magnesium deficient diet had a decrease in bacterial diversity as well as altered anxiety-like behavior (Jørgensen et al., 2014). In human subjects, associations studies demonstrated that intake of highly processed foods can decrease microbial diversity and was linked to increased risk for mental disorder (Dawson et al., 2016). Pro- and prebiotic supplementation are prominent ways to manipulate the microbiota and investigate the effect on

brain function. Supplementation with galactooligosaccharides stimulated the growth of *Bifidobacterium* and decreased waking cortisol response (Messaoudi et al., 2011) and probiotic supplementation with *Lactobacillus rhamnosus* GG in early childhood was protective against development of ADHD and Asperger Syndrome at 13 years of age (Pärtty et al., 2015). Due to the increasing knowledge of how GI microbes could potentially influence behavior in children with ASD, dietary interventions to manipulate the GI microbiota to ameliorate some symptoms of ASD could be a promising therapeutic avenue. Fro example, the purpose of the specific carbohydrate diet, which is used by some parents to manage symptoms of ASD, is to alleviate symptoms of malabsorption and prevent growth of potentially pathogenic bacteria which could potentially affect symptomology (Gotschall, 2004). Even though we did not find associations between a core symptom of ASD, social deficit scores, and diet-induced microbial profiles, scores for some GI symptoms were associated with dietary patterns and eating behavior, suggesting that some eating behaviors could potentially affect GI health through the microbiota in the ASD population. The commonly observed GI symptoms in the ASD population have been suggested to contribute to behavioral problems and to correlate with symptom severity (Tomova et al., 2015; Adams et al., 2011a; Buie et al., 2010; Coury et al., 2012a; McElhanon et al., 2014; Horvath et al., 1999); thus, promoting an eating pattern that promotes a microbial profile associated with fewer GI symptoms in the subpopulation of children with ASD and co-morbid GI problems might be a potential therapeutic avenue to alleviate some associated symptoms of ASD. However, future studies using more comprehensive GI assessment tools are needed to confirm the results reported herein.

The lack of association between social deficit scores and measures of diet-induced microbial profiles (i.e., feeding problems, dietary patterns, moderation analysis) could partly be

attributable to the small sample size. Likewise, it could be possible that repetitive or restrictive behaviors or other externalizing behaviors (i.e., aggression or irritability) associated with ASD could be more significantly impacted by the microbiota composition. Previous studies demonstrated that problematic eating behaviors could be a manifestation of repetitive behaviors, ritualizing or externalizing behaviors of ASD instead of social communication deficit (Johnson et al., 2014). Furthermore, one study investigating the relationship between individual bacterial taxa and symptoms of ASD found that *Desulfovibrio* was strongly correlated with the restricted/repetitive behavior subscale score (Tomova et al., 2015). Due to the age of the participants in this study, only social deficits were measured as the PDDBI-SV is one of the only validated parent questionnaire that is available for children under the age of 4. Likewise, it could be possible that other environmental factors contribute to the observed microbial dysbiois in ASD that could be associated with some symptoms of ASD. Thus, larger studies in the future should include measurements of all symptoms of ASD and consider other environmental factors known to influence the microbiota in order to provide more evidence for a relationship between diet-microbiota-symptoms in children with ASD.

Although the results presented in this study provide a new evidence for an impact of diet on microbiota in children with ASD, they are limited by the relatively small sample size of the population as well as the measurement of only social deficit symptoms of ASD. Additionally, dietary intake was based on parent self-report using food diaries and food frequency questionnaires, which are susceptible to measurement error and systemic biases in estimate food intake. Even though evidence for an association between food selectivity and ASD symptom severity is not conclusive, the reported observations could also be limited in that children with more severe ASD symptoms might be exert more picky eating behaviors or have more repetitive

eating patterns. Whereas some studies suggest that food selectivity and feeding problems were associated with higher rates of ASD symptoms as well as severity of temper tantrums (Crasta et al., 2014; Postorino et al., 2015; Dominick et al., 2007), other studies found no or limited association between feeding difficulties and severity of social, communication and cognitive deficits (Johnson et al., 2014; Tanner et al., 2015). It has been suggested that the severity of ASD symptoms might be related to topography and duration of refusal behavior, but not necessarily directly to food selectivity (Aponte et al., 2016). Using regression analysis, one study found that family food preferences instead of ASD symptom severity were the strongest predictors of child's food preferences (Schreck et al., 2006). Some of these discrepancies in study findings could be due to different definitions of food selectivity used in the various studies as well as the methodology used to assess feeding problem behaviors (Postorino et al., 2015). For example, food selectivity can refer to picky eating, food refusal, limited food repertoires, excessive intake of a few foods and selective intake of certain food categories (Cermak et al., 2010). Here, we report several instances in which social deficit scores were not related to eating behavior. First, we did not find any differences in children above or below the median in social deficit scores based on dietary patterns. Second, we did not see differences in social deficit scores based on picky eating behavior or repetitive eating patterns.

Despite these limitations, using various approaches new evidence for a relationship between diet and microbiota in ASD is reported, which have not previously been described in the ASD population. This approach could provide valuable insight for future studies aimed at deciphering the relationship between microbiota and ASD symptoms. Dietary intervention trials in children with ASD considering the baseline microbiota and investigating changes in the

microbiota that could relate to changes in some symptoms of ASD are needed to further elucidate if diet can be a modifiable moderator of GI microbiota- ASD symptoms relationship.

5.5 Tables

Table 5.1 Comparison of nutrient intake between children with ASD (ASD) and unaffected controls (CONT).

Variable	ASD (n=26)	CONT (n=32)	p-value
Macronutrients:			
Energy (kcal)	1349 (1103-1627)	1457 (1343-1734)	NS
Total Fat (g)	49 (36-58)	54 (43-63)	NS
Omega-6 Fatty Acids (g)	10.2 (7.5-13.3)	10.3 (8.5-12.6)	NS
Omega-3 Fatty Acids (g)	0.9 (0.8-1.3)	1.1 (0.8-1.3)	NS
SFA (g)	16 (12-22)	4 (2.9-5.0)	NS
MUFA (g)	17 (13-20)	18 (15-23)	NS
PUFA (g)	11 (8-15)	12 (9-14)	NS
Total Carbohydrate (g)	176 (147-220)	194 (159-225)	NS
Total Sugars (g)	81 (56-94)	84 (67-100)	NS
Added Sugars (g)	43 (28-49)	41 (32-50)	NS
Total Grains (oz equivalents)	5.1 (4.3-6.9)	5.9 (4.4-6.7)	NS
Whole Grains (oz equivalents)	0.7 (0.4-1.3)	1.0 (0.4-1.9)	NS
Refined Grains (oz equivalents)	4.4 (2.9-5.1)	4.2 (3.2-5.7)	NS
Total Protein (g)	49 (35-62)	52 (42-62)	NS
Dietary Fiber			
Total Dietary Fiber (g)	10 (9-17)	15 (11-17)	NS
Soluble Dietary Fiber (g)	3.5 (2.8-5.3)	4 (3-5)	NS
Insoluble Dietary Fiber (g)	6.8 (6-10.6)	9.8 (7.8-11.9)	0.09
Pectin (g)	1.3 (1.1-1.7)	1.9 (1.2-2.5)	0.09
Vitamins			
Vitamin A (μg)	435 (277-651)	369 (281-531)	NS
Vitamin D (μg)	3.1 (1.4-5.9)	4.4 (3.5-6.3)	NS
Vitamin E (mg)	6.2 (4.2-8.1)	5.1 (4.2-6.8)	NS
Vitamin K (μg)	33 (21-49)	44(35-56)	NS
Vitamin C (mg)	38 (29-57)	62 (46-115)	0.01
Thiamin (mg)	1.2 (0.9-1.4)	1.3 (1.0-1.6)	NS
Riboflavin (mg)	1.4 (1.1-1.8)	1.6 (1.3-1.9)	NS
Niacin (mg)	14 (12-19)	15 (11-19)	NS
Pantothenic Acid (mg)	3.5 (2.6-3.9)	3.5 (2.8-4.2)	NS
Vitamin B_6 (mg)	1.2 (0.9-1.5)	1.1 (0.9-1.5)	NS
Folate (μg)	238 (194-320)	276 (232-363)	NS
Vitamin B_{12} (μg)	3 (2.1-3.9)	3.2 (2.5-4.4)	NS
Choline (mg)	198 (135-247)	200 (150-253)	NS
Minerals			
Calcium (mg)	739 (451-958)	772 (586-980)	NS
Phosphorus (mg)	816 (599-1100)	930 (580-980)	NS
Magnesium (mg)	169 (131-231)	193 (174-227)	NS
Iron(mg)	9.9 (7.7-13.1)	9.9 (8.5-13.8)	NS

Table 5.1 (cont.)

Variable	ASD (n=26)	CONT (n=32)	p-value
Zinc (mg)	6.4 (4.5-7.6)	7.6 (6.1-8.7)	NS
Copper (mg)	0.7 (0.5-1.0)	0.8 (0.6-1.0)	NS
Selenium (mg)	64 (55-81)	72 (62-85)	NS
Sodium (mg)	1928 (1635-2245)	1983 (1548-2502)	NS
Potassium (mg)	1502 (1074-2170)	1796 (1446-1969)	NS
Manganese (mg)	1.9 (1.6-2.5)	2.4 (1.8-3.5)	NS

Data expressed as Median (IQR)

Table 5.2 Differences in intake of food groups between children with ASD (ASD) and unaffected controls (CONT).

Food Group	ASD (n=26)	CONT (n=32)	p-value
Fruit	2.1 (1.0-3.0)	2.0 (1.5-3.0)	NS
Vegetables	0.7 (0.3-1.71)	1.1 (0.7-1.9)	NS
Legumes, Nuts, Seeds	0.1 (0-0.4)	0.24 (0.16-0.44)	NS
Starchy Foods	0.2 (0.08-0.32)	0.2 (0.1-0.4)	NS
Starchy Vegetables	0.5 (0.2-0.9)	0.4 (0.3-0.6)	NS
Juice	0.8 (0-1.0)	0.14 (0.08-0.8)	NS
Sweetened Beverages	0 (0-0.08)	0.08 (0-0.16)	NS
Grains	0.7 (0.08-0.94)	0.7 (0.4-1.0)	NS
Refined Carbohydrates	0.9 (0.6-1.4)	0.9 (0.6-1.2)	NS
Fried Foods	0.2 (0.1-0.4)	0.2 (0.08-0.3)	NS
Protein	1.1 (0.4-1.8)	1.0 (0.7-1.6)	NS
Dairy	3.2 (1.8-4.0)	4.7 (3.1-5.5)	0.05
Snack	0.9 (0.5-1.6)	0.6 (0.4-1.0)	0.09
Sweets	1.7 (1.2-2.7)	1.4 (1.1-2.0)	0.1
Kid's Meal	0.7 (0.2-0.9)	0.8 (0.5-1.5)	NS
Fish	0 (0-0.1)	0.08 (0-0.2)	NS
Condiments	0.14 (0-0.4)	0.2 (0.08-0.6)	NS

Data expressed as Median (IQR)

Table 5.3 Differences in nutrient intake and microbiota composition between children with ASD characterized by picky eating behavior, including 20 foods in diet and repetitive eating pattern.

a. Picky Eating Behavior

Variable	Yes (n=13)	No (n=13)	p-value
Age (years)	4.6±1.5	3.6±1.3	NS
Gender (n)			
Female	2	5	NS
Male	9	10	
Weight (kg)	21.5±10.2	19.0±3.3	NS
Height (meters)	1.04±0.02	1.02±0.01	NS
BMI (kg/m^2)	17.1±3.1	17.4±1.9	NS
SOCDEF T-Score[1]	54±11	51±8	NS
GI severity score[2*]	3 (1.5-3)	1.5 (0-3)	NS
Constipation	1 (0-2)	0.5 (0-2)	NS
Diarrhea	0 (0-0)	0 (0-0)	NS
Stool Smell	0 (0-1)	0 (0-1)	NS
Flatulence	0 (0-0.5)	0.5 (0-1.5)	NS
Abdominal Pain	0.5 (0-1)	0 (0-0)	0.008
Nutrition*			
Nutrient intake[3]			
Total Fat (g)	45 (35-52)	55 (45-64)	0.09
Monounsaturated Fatty Acids (g)	13.5 (11.5-17.6)	18.2 (16.9-24.4)	0.06
Food groups[4]			
Juice (servings/day)	0.9 (0.8-1.0)	0.08 (0-0.8)	0.02
Protein Foods (servings/day)	0.8 (0.3-1.7)	1.6 (0.9-2.2)	0.06
Bacterial abundance* (% of sequences)			
Family			
Coriobacteriaceae	0.31 (0.02-1.12)	0.02 (0.002-0.05)	0.02
EtOH8	0 (0-0.06)	0 (0-0)	0.08
Genera			
Bacteroides	15.5 (8.6-29.22)	37.3 (29.6-47.0)	0.05
Ruminococcus	3.05 (1.9-6.9)	0.9 (0.17-2.1)	0.07
Holdemania	0.01 (0.002-0.02)	0.001 (0-0.007)	0.02
Phascolarctobacterium	0.03 (0.0008-0.09)	0.002 (0.0008-0.36)	0.09
VFA* (μmole/g DMB)			
Isobutyrate	7.82 (5.51-13.54)	5.78 (3.1-7.6)	0.08
Isovalerate	11.4 (6.3-20.4)	7.4 (4.5-11)	0.08

Data expressed as mean±SD; *data expressed as median (IQR)
[1]SOCDEF, social deficit score measured by PDDBI-SV
[2]GI severity scores were derived from the GI severity index (possible range 0-7)
[3]Nutrition intake was collected by 3-day food record
[4]Food groups derived from Youth and Adolescence Food Frequency Questionnaire
VFA, volatile fatty acids

Table 5.3 (cont.)

b. 20 Foods in diet

Variable	Yes (n=12)	No (n=14)	p-value
Age (years)	4.1±1.5	4.3±1.5	NS
Gender (n)			NS
Female	3	4	
Male	9	9	
Weight (kg)	17.7±3.6	22.9±9.9	NS
Height (meters)	1.02±0.02	1.07±0.02	NS
BMI (kg/m^2)	15.9±1.5	18.3±2.9	0.02
SOCDEF T-Score[1]	53±8	52±11	NS
GI Severity Score[2*]	1 (0-2)	3 (2-3)	0.01
Constipation	0 (0-2)	1 (0-2)	NS
Diarrhea	0 (0-0)	0 (0-0)	NS
Stool Smell	0 (0-0)	0 (0-1)	NS
Flatulence	0 (0-0)	1 (0-2)	0.02
Abdominal Pain	0 (0-0)	1 (0-1)	0.05
Nutrition*			
Nutrient Intake[3]			
Pectin (g)	1.4 (1.1-2.8)	1.2 (1.06-1.6)	0.08
Vitamin C (mg)	54 (33-98)	31 (24-41)	0.07
Niacin (mg)	16 (13-21)	12 (10-14)	0.01
Vitamin B_6 (mg)	1.2 (1.04-2.0)	1.14 (0.88-1.29)	0.05
Folate (μg)	243 (189-401)	226 (201-265)	0.06
Selenium (mg)	67 (60-80)	58 (45-84)	0.07
Added Sugars (g)	29 (24-39)	36 (22-47)	0.03
Bacterial abundance* (% of sequences)			
Phyla			
Actinobacteria	1.7 (0.7-2.9)	6.3 (3.1-13.6)	0.009
Bacteroidetes	55.2 (45.6-60.5)	39.7 (13.1-48.4)	0.03
Cyanobacteria	0.0007 (0-0.007)	0 (0-0)	0.05
Order			
Streptophyta	0.0007 (0-0.007)	0 (0-0)	0.02
Family			
Coriobacteriaceae	0.03 (0.005-0.07)	0.11 (0.007-0.9)	0.09
Clostridiales	2.66 (1.55-3.37)	3.61 (2.66-7.17)	0.02
Genus			
Bifidobacterium	0.96 (0.37-2.52)	2.58 (1.06-6.00)	0.06
Collinsella	0.02 (0.006-0.21)	1.21 (0.31-4.2)	0.05
Eggerthella	0.1 (0.05-0.37)	0.02 (0.01-0.05)	0.03
Bacteroides	40.2 (25.7-55.9)	25.8 (8.6-36.0)	0.08
Lactobacillus	0 (0-0)	0.001 (0-0.01)	0.09

Table 5.3 (cont.)

Dialister	0.62 (0.12-1.29)	0.008 (0.003-0.01)	0.04
Variable	**Yes (n=12)**	**No (n=14)**	**p-value**
VFA* (μmole/g DMB)			
Valerate	4.16 (1.08-8.6)	6.8 (4.7-8.1)	0.09

Data expressed as mean±SD; *data expressed as median (IQR)
[1]SOCDEF, social deficit score measured by PDDBI-SV
[2]GI severity scores were derived from the GI severity index (possible range 0-7)
[3]Nutrition intake was collected by 3-day food record
VFA, volatile fatty acids

Table 5.3 (cont.)

c. Repetitive Eating Behavior

Variable	Yes (n=14)	No (n=12)	p-value
Age (years)	4.4±1.6	3.7±1.1	NS
Gender (n)	3	4	NS
Female	13	6	
Male			
Weight (kg)	22.3±9.5	17.6±3.4	NS
Height (meters)	1.07±0.01	1.14±0.01	NS
BMI (kg/m^2)	18.1±2.8	15.8±1.4	0.03
SOCDEF T-Score[1]	54±8	51±10	NS
GI Severity Score[2*]	3 (2-3)	0.5 (0-2)	0.01
Constipation	1 (0-2)	0 (0-2)	NS
Diarrhea	0 (0-0)	0 (0-0)	NS
Stool Smell	0 (0-1)	0 (0-1)	NS
Flatulence	1 (0-2)	0 (0-0)	0.04
Abdominal Pain	0.5 (0-1)	0 (0-0)	0.01
Nutrition*			
Nutrient Intake[3]			
Pectin (g)	1.2 (1.0-1.6)	1.6 (1.2-2.9)	0.09
Vitamin C (mg)	33 (25-43)	84 (33-99)	0.02
Potassium (mg)	1340 (1034-1982)	1844 (1475-2394)	0.08
Copper (mg)	0.64 (0.49-0.75)	0.94 (0.75-1.24)	0.008
Bacterial abundance* (% of sequences)			
Phyla			
Actinobacteria	5.8 (1.5-14.5)	2.7 (1.0-3.04)	0.02
Verrucomicrobia	0.09 (0.02-0.72)	3.99 (0.02-6.7)	0.06
Cyanobacteria	0 (0-0)	0.001 (0-0.009)	0.07
Order			
Streptophyta	0 (0-0)	0 (0-0.003)	0.08
Family			
Clostridiales	3.6 (2.7-7.4)	1.9 (0.9-3.2)	0.09
Coriobacteriaceae	0.08 (0.02-0.76)	0.01 (0.002-0.39)	0.04
Genera			
Adlercreutzia	0.06 (0.04-0.19)	0.006 (0.001-0.39)	0.09
Collinsella	0.96 (0.14-4.26)	0.08 (0.005-0.25)	0.08
Eggerthella	0.03 (0.01-0.07)	0.14 (0.06-0.56)	0.06
Butyrivibrio	0.01 (0.005-0.03)	0.14 (0.003 (0-0.01)	0.04
Dialister	0.009 (0.003-0.44)	0.56 (0.01-0.9)	0.07
Coprobacillus	0 (0-0)	0.0004 (0-0.01)	0.09
Akkermansia	0.09 (0.02-0.72)	3.98 (0.02-6.71)	0.06

Data expressed as mean±SD; *data expressed as median (IQR)
[1]SOCDEF, social deficit score measured by PDDBI-SV

Table 5.3 (cont.)

[2]GI severity scores were derived from the GI severity index (possible range 0-7)
[3]Nutrition intake was collected by 3-day food record
VFA, volatile fatty acids

Table 5.4 Factor Loading Matrix for dietary patterns of three groups at baseline

a. ASD group

Food Group	Dietary Pattern 1	Dietary Pattern 2
Vegetables	0.88*	0.02
Starchy Vegetables	0.79*	0.16
Legumes, Nuts and Seeds	0.78*	0.08
Fruit	0.73*	0.13
Grains	0.56*	-0.002
Juice	0.45*	0.13
Dairy	0.36*	0.13
Fried Foods	0.13	0.67*
Kids Meals	-0.07	0.67*
Condiments	0.14	0.64*
Protein Foods	0.23	0.62*
Snacks	-0.33	0.59*
Starchy Foods	0.32	0.42*
Refined Carbohydrates	0.72*	0.50*
Fish	-0.03	0.21
Sweets	0.11	0.05
Sweetened Beverages	-0.29	0.26

b. CONT group

Food Group	Dietary Pattern 1	Dietary Pattern 2
Sweets	0.83*	0.05
Kid's Meals	0.82*	0.15
Fried Food	0.79*	0.07
Snacks	0.78*	-0.34
Starchy Foods	0.71*	-0.01
Dairy	0.54*	0.09
Sweetened Beverages	0.37*	0.24
Fish	-0.10	0.84*
Vegetables	-0.06	0.77*
Protein Foods	0.02	0.75*
Fruit	0.24	0.72*
Juice	0.24	0.44*
Refined Carbohydrates	0.46*	0.50*
Starchy Vegetables	0.25	0.34
Condiments	0.24	0.32
Grains	0.28	0.06
Legumes, Nuts and Seeds	-0.05	0.19

*Factor loading >0.35 is considered to be a major contributor to the overall dietary pattern; Food groups in which factor loadings are >0.35 for both dietary pattern are assigned to dietary pattern for which food group has highest factor loading contribution; dietary patterns are derived from YAQ data

Table 5.5 Participant characteristics by scores above or below the median in ASD group

	Dietary Pattern 1		Dietary Pattern 2	
Characteristic	Above Median (n=13)	Below Median (n=13)	Above Median (n=13)	Below Median (n=13)
Age	3.8±1.4	4.4±1.6	4.4±1.5	4±1.4
Gender (n)				
Male	11	9	10	9
Female	2	4	3	4
BMI (kg/m^2)	16.6±2.2	17.7±2.9	17.5±3	16.8±2.1
Race (n)				
Asian	0	2	2	0
African American	2	1	1	2
Hispanic	0	1	1	0
White	9	9	9	9
Other	2	0	0	2
Current Nutritional Supplement Use n (%)	6	5	6	5
Picky Eater n (%)	5	6	6	5
>20 foods in diet	8	4	4	8
Repetitive Eating Pattern n(%)	7	9	9	7
GI Severity Index*	1 (0-3)	3 (2-3)*	2 (1.5-3)	2 (0-4)
Constipation	0 (0-1)	2 (0-2)*	1.5 (0-2)	0.5 (0-1.5)
Diarrhea	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Stool Smell	0 (0-1)	0 (0-1)	0 (0-0.5)	0 (0-1)
Flatulence	0 (0-0.5)	0.5 (0-2)	0 (0-1)	0 (0-2)
Abdominal Pain	0 (0-1)	0 (0-0.5)	0 (0-0.5)	0 (0-1)
Bristol Stool Chart (n)				
Type 2	2	4	3	3
Type 3	3	2	3	2
Type 4	5	6	5	6
Type 5	2	0	1	1
Type 6	1	1	1	1

Table 5.5 (cont.)

SOCDEF T-score	52±10	52±9	50±6	54±12

Data are expressed as Mean ± SD or *n*; *data expressed as median (IQR); Values within same dietary pattern and row are significant at *p<0.05 and †p<0.1. GI, gastrointestinal; BMI, body mass index; SOCDEF, social deficit score

Table 5.6 Baseline food and nutrient intakes of participant in ASD group characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2

	Dietary Pattern 1		**Dietary Pattern 2**	
	Above Median (n=13)	Below Median (n=13)	Above Median (n=13)	Below Median (n=13)
Food Groups Intake (servings per day)				
Vegetables	1.6 (0.6-2.3)	0.3 (0.1-0.7)*	0.7 (0.2-1.2)	0. 6 (0.4-1.6)†
Legumes, Nuts, Seeds	0.4 (0-0.8)	0.1 (0.1-0.2)*	0 (0-0.2)	0.1 (0-0.4)*
Fruit	2.9 (1.9-3.6)	1.0 (0.5-2.3)*	2.3 (0.6-2.9)	1.9 (1.2-3.4)
Refined Carbohydrates	1.1 (0.9-1.7)	0.8 (0.4-0.9)*	0.9 (0.8-1.4)	0.9 (0.5-1.1)
Sweets	1.2 (0.9-1.8)	2.5 (1.6-3.1)†	2.0 (1.4-3.1)	1.7 (0.9-2.3)
Starchy Vegetables	0.9 (0.6-1.5)	0.2 (0.1-0.4)*	0.3 (0.1-0.5)	0.7 (0.4-1.2)*
Nutrient Intake				
Folate (µg)	235 (185-338)	242 (201-294)†	218 (193-265)	255 (203-338)
Vitamin E (mg)	6.5 (4.5-9.7)	5.8 (3.4-7.9)*	5.8 (4.2-7.8)	6.5 (4.5-9.5)
Vitamin B_{12} (mg)	2.9 (2.2-3.8)	3.0 (2.1-4.0)*	3.0 (2.8-3.4)	2.5 (3.1-4.0)†
Vitamin A (mg)	534 (442-712)	326 (278-428)*	413 (282-565)	442 (278-651)
Niacin (mg)	14.7 (13.5-16.7)	12.4 (20.6-18.9)	12.4 (12.0-14.7)	16.7 (13.8-20.3)†
Vitamin B_6 (mg)	1.2 (0.9-1.3)	1.1 (0.9-1.8)	1.1 (0.9-1.3)	1.3 (0.9-1.9)†
Total Grains (g)	5.3 (4.3-6.7)	5.0 (4.3-7.4)	4.7 (3.8-5.5)	5.9 (4.8-7.3)*
Refined Grains (g)	3.9 (2.6-4.8)	4.6 (3.7-5.1)	4.3 (2.8-5.0)	4.6 (3.7-5.4)†
Total Dietary Fiber (g)	11 (9.8-16.9)	9.6 (7.3-14.8)	9.8 (9.3-12.1)	10.9 (9.2-16.9)
Soluble Dietary Fiber (g)	4.9 (3.1-5.4)	3.6 (2.4-4.3)	3.3 (2.4-5.2)	3.5 (3.1-5.4)
Insoluble Dietary Fiber (g)	7.3 (6.4-10.6)	6.3 (4.9-10.5)†	6.5 (6.1-7.3)	7.4 (6.0-10.6)
Pectin (g)	1.2 (1.1-1.8)	1.5 (1.1-1.7)	1.3 (1.1-1.7)	1.3 (0.9-1.8)

Data are expressed as median (IQR); Values within same pattern and row are significant at *$p<0.05$ and †$p<0.1$; Only nutrient intake that showed significant difference between the groups are shown; Food groups intake derived from YAQ; Nutrient intake derived from 3-day food record

Table 5.7 Baseline bacterial abundance and VFA concentrations of participants in ASD group characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2

	Dietary Pattern 1		**Dietary Pattern 2**	
Characteristic	Above Median (n=13)	Below Median (n=13)	Above Median (n=13)	Below Median (n=13)
Bacterial Abundance (% of sequences)				
Family	0.07 (0.004-0.07)	0.16 (0.008-0.12)†	0.17 (0.008-0.12)	0.06 (0.005-0.1)
Enterobacteriaceae	0.9 (0.006-1.45)	2.83 (0.006-2.98)	3.0 (0.008-2.85)	0.73 (0.006-0.97)*
Barnesiellaceae	0.01 (0-0.02)	0.006 (0-0.004)	0.003 (0-0.004)	0.003 (0-0.005)†
Streptophyta				
Genera	0.003 (0-0.005)	0.01 (0.002-0.02)*	0.006 (0-0.008)	0.009 (0.006-0.01)
Lactococcus	0.38 (0.12-0.41)	0.58(0.18-0.57)†	0.46 (0.14-0.36)	0.50 (0.22-0.51)
Roseburia	0 (0-0)	0.002 (0-0.002)†	0.0002 (0-0)	0 (0-0)
Leuconostoc	2.14 (0.89-1.78)	2.8 (0.9-3.83)†	1.6 (0.43-1.78)	3.33 (1.21-3.83)
Ruminococcus	0.01 (0-0.02)	0.02 (0-0.01)	0.03 (0-0.04)	0.006 (0-0.004)†
Alistipes				
VFA Concentration (μmol/g)	155 (110-255)	173 (131-243)	200 (131-255)	143 (111-246)
Acetate	51 (39-76)	55 (51-90)	68 (51-90)	50 (39-61)†
Propionate	49 (37-74)	66 (42-76)	67 (42-76)	48 (37-74)
Butyrate	6.2 (4.25-7.7)	7.8 (4.5-10)	9.4 (6.2-11.5)	5.4 (3.6-7.2)*
Isobutyrate	4.4 (1.4-7.9)	6.8 (4.7-8.1)	7.9 (6.5-10.8)	4.2 (1.2-5.9)*
Valerate	8.5 (5.5-11)	11.7 (6.3-15)	13.2 (8-15.5)	6.7 (5.5-10.6)*
Isovalerate				

Data are expressed as Median (IQR); Values within same dietary pattern and row are significant at *p<0.05 and †p<0.1; VFA, volatile fatty acids

Table 5.8 Participant characteristics by scores above or below the median in CONT group

Characteristic	Dietary Pattern 1		Dietary Pattern 2	
	Above Median (n=15)	Below Median (n=16)	Above Median (n=15)	Below Median (n=16)
Age	3.5±1.0	4.1±1.0	3.7±0.9	3.9±1.2
Gender (n)				
Male	8	5	10	8
Female	5	10	5	8
BMI (kg/m^2)	17±2.5	16.3±2.7†	16.8±2.6	16.44±2.6
Race (n)				
Asian	3	2	4	1
African American	1	2	1	2
Hispanic	1	1	0	2
White	8	11	10	9
Other	2	0	0	2
Current Nutritional Supplement Use n (%)	6	7	5	8
Picky Eater n (%)	3	7	5	5
>20 foods in diet	14	14	14	14
Repetitive Eating Pattern n(%)	5	8	5	8
GI Severity Index*	0 (0-1)	1 (0-2)	1 (0-2)	0 (0-1.5)
Bristol Stool Chart (n)				
Type 2	0	1	1	0
Type 3	7	6	4	9
Type 4	9	7	9	7

Data are expressed as Mean ± SD or *n*; *data expressed as median (IQR); Values within same dietary pattern and row are significant at *$p<0.05$ and †$p<0.1$; GI, gastrointestinal; BMI, body mass index

Table 5.9 Baseline food and nutrient intakes of participant in CONT group characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2.

	Dietary Pattern 1		**Dietary Pattern 2**	
	Above Median (n=16)	Below Median (n=16)	Above Median (n=16)	Below Median (n=16)
Food Groups Intake (servings per day)				
Fruit	2.9 (1.9-3.4)	1.9 (1.3-2.2)	2.7 (1.9-3.6)	1.7 (1.2-2.0)*
Refined Carbohydrates	1.2 (0.9-1.4)	0.7 (0.4-1.0)*	1.1 (0.7-1.4)	0.8 (0.4-1.1) †
Sweets	1.9 (1.6-2.5)	1.1 (0.6-1.3)*	1.4 (1.1-2.1)	1.4 (0.8-1.7)
Starchy Vegetables	0.4 (0.3-0.6)	0.4 (0.4-0.5)	0.4 (0.3-1.0)	0.4 (0.2-0.4)*
Protein Foods	0.9 (0.7-1.5)	1.0 (0.8-1.3)	1.3 (0.8-2.0)	0.7 (0.4-1.2)*
Snacks	0.9 (0.7-1.5)	0.5 (0.2-0.6)*	0.5 (0.4-0.8)	0.8 (0.3-1.4)
Fish	0.04 (0.0-	0.2 (0-0.2)	0.2 (0.1-0.3)	0 (0-0)*
Dairy Foods	0.2)	3.9 (2.2-5.0)†	4.9 (2.3-6.6)	4.1 (3.3-5.1)
Kid's Meals	4.9 (4.2-5.9)	0.6 (0.3-0.9)*	0.7 (0.6-1.5)	0.9 (0.3-1.3)
Grain Foods	1.4 (0.7-1.6)	0.6 (0.4-1.0)	0.7 (0.4-1.2)	0.6 (0.4-0.9)†
	0.6 (0.4-0.7)			
Nutrient Intake				
Total Fat (g)	54 (39-63)	54 (47-68)	58 (49-76)	52 (41-57)†
Saturated Fatty Acids (g)	17 (13-23)	20 (16-24)	22 (16-25)	18 (13-21)*
Omega-3 Fatty Acids (g)	1.1 (0.8-1.3)	1.2 (0.9-1.4)	1.3 (1.1-1.4)	0.9 (0.8-1.2)†
Vitamin B_{12} (mg)	2.8 (2.0-3.6)	3.5 (3.2-5.2)*	3.4 (2.7-4.4)	3.2 (2.5-4.0)
Zinc (mg)	6.7 (5.9-7.9)	7.8 (7.1-11.4)*	7.1 (5.9-10.5)	7.8 (7.0-8.7)
Sodium (mg)	1773 (1380-2458)	2172 (1760-2652)	2295 (1524-2811)	1892 (1538-2376)*
Refined Grains	0.8 (0.4-1.9)	1.1 (0.4-2.0)	0.7 (02-1.1)	1.6 (0.9-1.4)*
Total Dietary Fiber (g)	13 (10-17)	15 (13-18)	15 (7-17)	14 (13-18)
Soluble Dietary Fiber (g)	3.5 (2.3-4.8)	4.9 (3.9-5.2)*	3.8 (2.7-4.9)	4.5 (3.3-5.3)
Insoluble Dietary Fiber (g)	9.8 (7.7-12.1)	10 (8.4-12.5)	9.8 (5.0-11.9)	10 (8.5-12.6)
Pectin (g)	1.6 (0.8-2.2)	2.3 (1.6-2.8)†	2.0 (0.8-2.5)	1.9 (1.4-2.5)

Table 5.9 (cont.)

Data are expressed as Median (IQR); Values within same pattern and row are significant at *p<0.05 and †p<0.1; Only nutrient intake that showed significant difference between the groups are shown; Food groups intake derived from YAQ; Nutrient intake derived from 3-day food record

Table 5.10 Baseline bacterial abundance and VFA concentrations of participants in CONT group characterized as consuming above or below the median in dietary pattern 1 and dietary pattern 2

	Dietary Pattern 1		**Dietary Pattern 2**	
Characteristic	Above Median (n=13)	Below Median (n=13)	Above Median (n=13)	Below Median (n=13)
Bacterial Abundance (% of sequences)				
Phyla				
Cyanobacteria	0.004 (0-0.02)	0 (0-0.005)	0.003 (0-0.005)	0.0008(0-0.02)†
Verrucomicrobia	0.4 (0.01-2.99)	0.35 (0.01-14.49)	0.03 (0.01-1.9)	2.4 (0.08-15.6)†
Family				
Rikenellaceae	2.86 (1.15-7.44)	3.02 (0.53-6.32)	4.6 (0.9-7.4)	2.4 (0.8-5.1)†
Streptophyta	0.005 (0-0.02)	0 (0-0.004)	0.003 (0-0.005)	0.008 (0-0.01)†
Enterobacteriaceae	0.01 (0.004-0.07)	0.03. (0.007-0.12)	0.08 (0.02-0.14)	0.008 (0.003-0.02)*
Genera				
Eubacterium	0.03 (0.01-0.1)	0.02 (0.01-0.03)†	0.02 (0.009-0.04)	0.03 (0.02-0.07)
Coprobacillus	0.001 (0-0.01)	0 (0-0.002)	0 (0-0.01)	0 (0-0.005)*
Akkermansia	0.4 (0.01-2.99)	0.35 (0.01-14.49)	0.03 (0.01-1.9)	2.4 (0.08-15.6)†
VFA Concentration (μmol/g)				
Acetate	132 (101-175)	98 (45-181)	113 (93-175)	126 (58-173)
Propionate	51 (44-70)	38 (14-60)	48 (37-70)	46 (26-62)
Butyrate	39 (21-80)	27 (11-60)	27 (21-103)	36 (12-53)
Isobutyrate	8.6 (4.5-10.6)	3.7 (3.1-5.8)	4.5 (3.5-10.4)	5.3 (3.1-7.4)
Valerate	6.8 (2.9-9.5)	2.5 (1.2-5.5)*	2.9 (1.1-8.5)	4.3 (2.3-6.8)
Isovalerate	12.2 (5.9-15.7)	5.7 (4.6-8.1)	6.7 (5.2-15.3)	6.8 (4.6-10.2)

Data are expressed as Median (IQR); Values within same dietary pattern and row are significant at *$p<0.05$ and †$p<0.1$; VFA, volatile fatty acids

Table 5.11 Summary of moderation analysis procedure

a) Fecal microbiota predict SOCDEF scores (dependent variable)

Step	Variable Entered	Variable Removed	No. of variables included	Partial R^2	Model R^2	C(p)	F Value	P-value
1	*Faecalibacterium*	None	1	0.2669	0.2669	26.3313	8.01	0.0097
2	*Lactobacillus*	None	2	0.1748	0.4418	17.2805	6.58	0.0181
3	Peptostreptococcaceae	None	4	0.0933	0.6581	7.6087	5.19	0.0345
4	*Dialister*	None	5	0.0571	0.7152	6.0000	3.61	0.0736

Fecal microbiota (independent variable) presented as relative abundance

b) Dietary factors predict SOCDEF scores (dependent variable).

Step	Variable Entered	Variable Removed	No of Variables Included	Partial R^2	Model R^2	C(p)	F Value	P-value
1	Total Grain	None	1	0.35	0.35	9.37	11.77	0.002
2	Calcium	None	2	0.12	0.47	5.59	4.75	0.04

Total grain and calcium intake were derived form 3-day food record

Table 5.12 Hierarchical regression analysis testing for potential moderating role of dietary factors in the relationship between microbiota and SOCDEF scores

Outcome Variable	β	ΔR^2	p-value	95% CI
Total Grains Intake	1.0		0.005	1.1,5.3
Faecalibacterium abundance	0.62		0.1	-0.26, 2.36
Total Grains x *Faecalibacterium* abundance	0.08	0.04	0.24	-0.26,0.07
Total Grains Intake	0.87		0.002	1.31, 4.94
Peptostreptococcaceae Abundance	20.32		0.91	-40.25, 44.80
Total Grains Intake x Peptostreptococcaceae abundance	2.84	0.005	0.69	-7.11, 4.78
Calcium Intake	0.01		0.80	-0.02, 0.03
Faecalibacterium abundance	0.92		0.14	-0.5, -3.3
Calcium x *Faecalibacterium* abundance	0.001	0.03	0.38	-0.01,0.01
Calcium Intake	0.008		0.55	-0.02, 0.01
Peptostreptococcaceae Abundance	42.77		0.16	-26.98, 152.09
Calcium Intake x Peptostreptococcaceae abundance	0.04	0.07	0.23	-0.13, 0.03

CI= Confidence Interval; All regression coefficients are unstandardized; all predictors were mean centered prior to entering into the regression model

5.6 Supplementary Tables

Supplemental Table 5.1 Correlations between Nutrients and Food groups and microbiota

a) ASD

Nutrient Intake							
Energy	Slackia	Bacteroidales	Butyricimonas	Megamonas	RF32	Akkermansia	Verrucomicrobia
ρ	0.37	0.43	-0.40	0.39	-0.42	-0.45	-0.45
p-value	0.06	0.03	0.04	0.05	0.03	0.02	0.02

Total Fat	Bacteroidales	Alistipes	Dialister
ρ	0.34	-0.34	0.35
p-value	0.09	0.09	0.08

Total Carbohydrate	Slackia	Bacteroidales	Parabacteroides	Dialister	Megamonas	Phascolarctobacterium	Coprobacillus	RF32	Enterobacteriaceae	Akkermansia	Verrucomicrobia
ρ	0.35	0.34	0.36	0.44	0.34	-0.38	-0.38	-0.42	-0.35	-0.46	-0.46
p-value	0.08	0.09	0.07	0.02	0.09	0.06	0.06	0.03	0.08	0.02	0.02

Total Protein	Bacteroidales	Barnesiellaceae	Butyricimonas	Staphylococcus	Lachnospiraceae	Coprococcus	Peptococcus	Pep_Clostridium	RF32
ρ	0.54	-0.35	-0.40	-0.41	-0.34	-0.51	0.51	0.54	-0.43
p-value	0.00	0.08	0.04	0.04	0.09	0.01	0.01	0.00	0.03

Starch	Bacteroidales	Odoribacter	Lactococcus	SMB53	Ruminococcaceae	Anaerotruncus	Faecalibacterium	Coprobacillus	RF39	Akkermansia	Tenericutes	Verrucomicrobia
ρ	0.34	-0.34	0.36	0.43	0.35	-0.45	0.36	-0.39	0.40	-0.49	0.40	-0.49
p-value	0.08	0.09	0.07	0.03	0.08	0.02	0.07	0.05	0.04	0.01	0.04	0.01

Supplemental Table 5.1 (cont.)

Total Dietary Fiber	Bacteroidales	Parabacteroides	Staphylococcus	Blautia	Coprococcus	Dorea	Oscillospira	Dialister	Megamonas	Phascolarctobacterium	RF32	Bilophila	Enterobacteriaceae
ρ	0.38	0.34	-0.45	-0.45	-0.42	-0.33	0.34	0.35	0.35	-0.35	-0.38	-0.38	-0.39
p-value	0.05	0.09	0.02	0.02	0.03	0.10	0.09	0.08	0.08	0.08	0.05	0.06	0.05

Soluble Dietary Fiber	Bacteroidales	Odoribacter	Dialister	Megamonas	Phascolarctobacterium	Erysipelotrichaceae	Catenibacterium	Coprobacillus	RF32	Bilophila	Akkermansia	Verrucomicrobia
ρ	0.37	-0.40	0.38	0.47	-0.40	-0.36	0.42	-0.43	-0.35	-0.36	-0.37	-0.37
p-value	0.06	0.04	0.06	0.01	0.04	0.07	0.03	0.03	0.08	0.07	0.07	0.07

Insoluble Dietary Fiber	Bifidobacterium	Parabacteroides	Staphylococcus	Clostridiales	Lachnospiraceae	Blautia	Coprococcus	Dorea	Oscillospira	RF32	Bilophila	Enterobacteriaceae	Actinobacteria	Bacteroidetes
ρ	-0.43	0.33	-0.62	-0.40	-0.35	-0.46	-0.55	-0.41	0.39	-0.39	-0.40	-0.36	-0.44	0.34
p-value	0.03	0.10	0.00	0.04	0.08	0.02	0.00	0.04	0.05	0.05	0.04	0.07	0.03	0.09

Pectins	Bacteroides	Parabacteroides	Staphylococcus	Lactococcus	Clostridiaceae_family	2d06	SMB53	Blautia	Lachnospira	Mogibacteriaceae	Erysipelotrichaceae_Eubacterium	RF32	Actinobacteria	Bacteroidetes
ρ	0.36	0.45	-0.42	-0.34	-0.35	-0.53	-0.39	-0.40	0.49	-0.45	-0.45	-0.48	-0.34	0.39
p-value	0.07	0.02	0.03	0.09	0.08	0.01	0.05	0.04	0.01	0.02	0.02	0.01	0.09	0.05

Supplemental Table 5.1 (cont.)

Total Grains	Lactococcus	Clostridiaceae_family	2d06	Clostridium	SMB53	Lachnospiraceae	Peptostreptococcaceae	Ruminococcaceae	Anaerotruncus	RF39	Akkermansia	Firmicutes	Tenericutes	Verrucomicrobia
ρ	0.42	0.42	0.37	0.35	0.55	0.34	0.45	0.41	-0.49	0.54	-0.40	0.37	0.54	-0.40
p-value	0.03	0.03	0.07	0.08	0.00	0.09	0.02	0.04	0.01	0.00	0.04	0.06	0.00	0.04

Whole Grains	Bifidobacterium	Adlercreutzia	Slackia	Bacteroidales	Paraprevotellaceae__Prevotella	02d06	Anaerostipes	Lachnospiraceae_Ruminococcus	Peptococcus	Mogibacteriaceae	Catenibacterium	Coprobacillus	Campylobacter	Proteobacteria
ρ	-0.36	0.41	0.43	0.57	0.40	0.35	0.39	-0.45	0.46	0.42	0.41	-0.39	-0.46	-0.37
p-value	0.07	0.04	0.03	0.00	0.04	0.08	0.05	0.02	0.02	0.03	0.04	0.05	0.02	0.06

Refined Grains	Slackia	Parabacteroides	EtOH8	Lachnospiraceae	Butyrivibrio	Peptococcus	Mogibacteriaceae	Catenibacterium	Campylobacter	RF39	Akkermansia	Tenericutes	Verrucomicrobia
ρ	-0.39	-0.40	-0.45	0.56	0.52	-0.53	-0.33	-0.51	0.46	0.47	-0.45	0.47	-0.45
p-value	0.05	0.04	0.02	0.00	0.01	0.01	0.10	0.01	0.02	0.01	0.02	0.01	0.02

Food Groups Intake

Fruit	S247	Faecalibacterium
ρ	0.33	-0.39
p-value	0.10	0.05

Vegetables	Bifidobacterium	Bacteroides	SMB53	Faecalibacterium	Acidaminococcus	Holdemania	Actinobacteria	Bacteroidetes	Firmicutes
ρ	-0.56	0.42	-0.37	-0.38	0.35	-0.47	-0.44	0.43	-0.46

Supplemental Table 5.1 (cont.)

p-value	0.00	0.04	0.06	0.06	0.08	0.02	0.02	0.03	0.02					
Legumes	Bifidobacterium	Slackia	Bacteroides	Staphylococcus	Enterococcus	Leuconostoc	Lactococcus	Turicibacter	SMB53	Peptostreptococcaceae	Acidaminococcus	Holdemania	Actinobacteria	Bacteroidetes
ρ	-0.40	-0.35	0.56	-0.41	-0.38	-0.35	-0.42	-0.52	-0.55	-0.42	0.36	-0.58	-0.55	0.49
p-value	0.04	0.08	0.00	0.04	0.05	0.08	0.03	0.01	0.00	0.03	0.07	0.00	0.00	0.01
Starchy Foods	Odoribacter	EtOH8	Lachnospiraceae	Lachnospiraceae_Ruminococcus	Megamonas	Mogibacteriaceae	Fusobacterium	Campylobacter	Akkermansia	Fusobacteria	Verrucomicrobia			
ρ	0.35	0.38	-0.35	-0.49	-0.38	0.37	-0.34	-0.34	0.46	-0.34	0.46			
p-value	0.08	0.06	0.08	0.01	0.06	0.07	0.09	0.08	0.02	0.09	0.02			
Starchy Vegetables	Bifidobacterium	S247	Lactobacillus	Ruminococcaceae	Enterobacteriaceae	Actinobacteria	Bacteroidetes							
ρ	-0.56	0.33	-0.34	0.41	-0.35	-0.53	0.33							
p-value	0.00	0.10	0.09	0.04	0.08	0.01	0.09							
Juice	Prevotella	RF16	Alistipes	S247	Barnesiellaceae	Paraprevotella	Paraprevotellaceae_Prevotella	Enterobacteriaceae						
ρ	0.35	0.40	0.56	0.34	0.53	0.50	0.55	-0.35						
p-value	0.08	0.04	0.00	0.09	0.01	0.01	0.00	0.08						
Sweetened Beverages	Coriobacteriaceae	Adlercreutzia	Collinsella	Rikenellaceae	Odoribacter	Ruminococcus	Veillonella	Mogibacteriaceae	Erysipelotrichaceae	Bilophila	Haemophilus			
ρ	0.44	0.44	0.34	0.49	0.42	0.52	-0.63	0.53	0.43	0.54	-0.43			
p-value	0.02	0.02	0.09	0.01	0.03	0.01	0.00	0.01	0.03	0.00	0.03			

Supplemental Table 5.1 (cont.)

Grains	S247	Streptococcus	Dialister	Coprobacillus
ρ	0.34	-0.34	0.45	-0.41
p-value	0.09	0.09	0.02	0.04

Refined Carbohydrates	Streptococcus	Holdemania
ρ	-0.39	-0.42
p-value	0.05	0.03

Fried Food	Eggerthella	Alistipes	Turicibacter	Pep_Clostridium	Faecalibacterium	Acidaminococcus	Erysipelotrichaceae_Eubacterium	Sutterella	Enterobacteriaceae
ρ	-0.41	0.43	-0.33	-0.40	0.43	0.46	-0.41	0.39	-0.43
p-value	0.04	0.03	0.09	0.04	0.03	0.02	0.04	0.05	0.03

Protein	Bacteroides	Prevotella	Rikenellaceae	Turicibacter	SMB53	Peptostreptococcaceae	Oscillospira	Acidaminococcus	Megamonas	Holdemania	Firmicutes
ρ	0.50	-0.36	0.34	-0.36	-0.43	-0.39	-0.36	0.54	-0.48	-0.39	-0.38
p-value	0.01	0.07	0.09	0.07	0.03	0.05	0.07	0.00	0.01	0.05	0.06

Dairy	Megamonas
ρ	0.40
p-value	0.04

Snacks	Coriobacteriaceae	Eggerthella	Bacteroides	Parabacteroides	Rikenellaceae	Alistipes	Barnesiellaceae	Coprococcus	Roseburia	Peptococcus	Pep_Clostridium	Faecalibacterium	Ruminococcus	Megasphaera
ρ	0.41	-0.40	-0.47	0.33	0.38	0.61	0.49	0.39	0.47	-0.44	-0.64	0.34	0.45	0.35
p-value	0.04	0.04	0.02	0.10	0.06	0.00	0.01	0.05	0.02	0.02	0.00	0.09	0.02	0.08

Sweets	Bacteroides	Prevotella	RF16	Alistipes	Odoribacter	Blautia	Peptococcus	Ruminococcaceae	Faecalibacterium
ρ	-0.35	0.42	0.38	0.38	0.34	0.35	-0.36	-0.36	0.33
p-value	0.08	0.03	0.05	0.06	0.09	0.08	0.07	0.07	0.09

Supplemental Table 5.1 (cont.)

Kids Meal	Coriobacteriaceae	Adlercreutzia	Collinsella	Paraprevotella	Coprococcus	Roseburia	Acidaminococcus	Megasphaera	Mogibacteriaceae	Erysipelotrichaceae	Haemophilus	Proteobacteria
ρ	0.38	0.42	0.49	0.36	0.38	0.50	0.33	0.39	0.34	0.37	-0.43	-0.54
p-value	0.06	0.03	0.01	0.07	0.06	0.01	0.10	0.05	0.09	0.06	0.03	0.00

Fish	Bacteroides	Turicibacter	Clostridium	Pseudoramibacter_Eubacterium	Lachnospira	Oscillospira	Fusobacterium	Fusobacteria
ρ	0.34	-0.34	-0.35	-0.35	-0.36	-0.33	-0.38	-0.38
p-value	0.09	0.09	0.08	0.08	0.07	0.10	0.05	0.05

Condiments	Eggerthella	Megasphaera	Veillonella	Fusobacterium	RF32	Haemophilus	Fusobacteria
ρ	-0.34	0.47	-0.37	-0.48	-0.36	-0.39	-0.48
p-value	0.09	0.01	0.07	0.01	0.07	0.05	0.01

Supplemental Table 5.1 (cont.)
b) CONT

Nutrient Intake										
Energy	Odoribacter	Paraprevotella	Lactobacillus	Turicibacter	Peptococcus	Erysipelotrichaceae_Eubacterium	Sutterella	RF39	Proteobacteria	Tenericutes
ρ	0.42	0.37	-0.33	-0.38	-0.36	-0.45	0.46	-0.33	0.40	-0.33
p-value	0.02	0.05	0.08	0.04	0.05	0.01	0.01	0.07	0.03	0.07

Total Fat	Adlercreutzia	Eggerthella	Paraprevotella	Clostridium	Anaerostipes	Ruminococcaceae	Phascolarctobacterium	Succiniclasticum	Erysipelotrichaceae_Eubacterium	Fusobacterium	RF39	Fusobacteria	Tenericutes
ρ	0.36	-0.33	0.36	0.34	0.31	0.31	0.44	0.34	-0.31	-0.33	-0.47	-0.33	-0.47
p-value	0.05	0.08	0.05	0.07	0.09	0.09	0.01	0.07	0.10	0.07	0.01	0.07	0.01

TotalCarbohydrate	Collinsella	Bacteroides	RF16	Lactococcus	Anaerostipes	Dorea	Ruminococcaceae	Sutterella	Proteobacteria
ρ	-0.38	0.34	-0.31	-0.42	-0.42	-0.34	-0.33	0.40	0.34
p-value	0.04	0.07	0.10	0.02	0.02	0.07	0.08	0.03	0.07

TotalProtein	Coriobacteriaceae	Adlercreutzia	Collinsella	Prevotella	Alistipes	Barnesiellaceae	Clostridiales	SMB53	Coprococcus	Roseburia	Ruminococcaceae	Succiniclasticum	Coprobacillus	Erysipelotrichaceae_Eubacterium	Enterobacteriaceae	RF39	Tenericutes
ρ	0.42	0.38	0.35	-0.33	0.31	0.36	0.41	0.39	0.33	0.34	0.35	0.32	-0.38	-0.32	0.37	-0.40	-0.40
p-value	0.02	0.04	0.06	0.08	0.09	0.05	0.02	0.03	0.08	0.07	0.06	0.08	0.04	0.08	0.04	0.03	0.03

Supplemental Table 5.1 (cont.)

Starch	Odoribacter	Lactobacillus	Lactococcus	Anaerostipes	Anaerotruncus	Succiniclasticum	RF32	Sutterella
ρ	0.33	-0.37	-0.48	-0.41	-0.36	-0.34	-0.35	0.36
p-value	0.08	0.04	0.01	0.02	0.05	0.06	0.06	0.05

TotalDietaryFiber	Turicibacter	Dorea	Lachnospira	Peptococcus	Anaerotruncus	Ruminococcus	Erysipelotrichaceae_Eubacterium	Sutterella
ρ	-0.47	-0.31	0.46	-0.33	-0.52	-0.43	-0.61	0.43
p-value	0.01	0.10	0.07	0.00	0.02	0.00	0.02	0.03

Soluble Dietary Fiber	Turicibacter	Christensenellaceae	Anaerostipes	Anaerotruncus	Erysipelotrichaceae_Eubacterium	Sutterella	Proteobacteria
ρ	-0.38	-0.34	-0.31	-0.54	-0.53	0.31	0.35
p-value	0.04	0.07	0.09	0.00	0.00	0.09	0.06

InsolubleDietaryFiber	Eggerthella	Turicibacter	Dorea	Lachnospira	Lachnospiraceae_Ruminococcus	Peptococcus	Anaerotruncus	Ruminococcus	Veillonella	Catenibacterium	Erysipelotrichaceae_Eubacterium	Sutterella	Haemophilus	Proteobacteria
ρ	-0.13	-0.43	-0.32	0.53	-0.37	-0.32	-0.49	-0.39	-0.32	-0.09	-0.56	0.49	-0.04	0.43
p-value	0.50	0.02	0.09	0.00	0.05	0.09	0.01	0.03	0.09	0.64	0.00	0.01	0.84	0.02

Pectins	Alistipes	Turicibacter	Anaerostipes	Coprococcus	Anaerotruncus	Ruminococcus	Erysipelotrichaceae_Eubacterium
ρ	0.31	-0.42	-0.35	0.20	-0.38	-0.32	-0.48
p-value	0.10	0.02	0.06	0.28	0.04	0.08	0.01

Supplemental Table 5.1 (cont.)

Total Grains	Collinsella	Bacteroides	Lactococcus	Christensenellaceae	Lachnospiraceae	Blautia	Ruminococcaceae	RF32	Sutterella	Bilophila	Campylobacter	Enterobacteriaceae	Haemophilus	RF39	Akkermansia	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria	Tenericutes	Verrucomicrobia
ρ	-0.31	0.31	-0.44	-0.35	-0.07	0.01	-0.30	-0.45	0.36	0.08	0.23	-0.21	-0.05	0.09	0.06	-0.26	0.11	-0.06	0.15	0.24	0.09	0.06
p-value	0.10	0.09	0.02	0.06	0.73	0.97	0.10	0.01	0.05	0.68	0.23	0.27	0.80	0.65	0.75	0.17	0.56	0.74	0.42	0.21	0.65	0.75

WholeGrains	Pseudoramibacter_Eubacterium	Anaerotruncus	Phascolarctobacterium
ρ	0.31	-0.35	-0.33
p-value	0.09	0.06	0.08

RefinedGrains	Lactobacillus	Faecalibacterium	RF32
ρ	-0.39	-0.01	-0.44
p-value	0.03	0.97	0.01

Fruit	Pseudoramibacter_Eubacterium	Dialister
ρ	-0.35	0.41
p-value	0.06	0.03

Vegetables	Bacteroides	Lactobacillus	EtOH8	Pseudoramibacter_Eubacterium	Catenibacterium	Erysipelotrichaceae_Eubacterium	Bacteroidetes
ρ	0.32	-0.47	-0.33	-0.53	0.39	-0.32	0.31
p-value	0.09	0.01	0.07	0.00	0.03	0.08	0.09

Supplemental Table 5.1 (cont.)

Legumes, nuts and seeds	Bacteroides	Prevotella	RF16	Alistipes	EtOH8	Blautia	Lachnospira	Ruminococcus	Succiniclasticum	Veillonella	Haemophilus	Akkermansia	Verrucomicrobia
ρ	0.47	-0.46	-0.32	-0.31	-0.55	-0.32	0.33	-0.31	0.36	0.35	0.45	-0.40	-0.40
p-value	0.01	0.01	0.08	0.09	0.00	0.09	0.07	0.09	0.05	0.05	0.01	0.03	0.03

Starchy Foods	Coriobacteriaceae	Bacteroides	Alistipes	Paraprevotella	Paraprevotellaceae__Prevotella	Clostridiaceae_family	SMB53	Blautia	Peptostreptococcaceae	Erysipelotrichaceae	Catenibacterium	RF39	Bacteroidetes	Tenericutes
ρ	-0.31	0.50	-0.42	-0.42	0.32	-0.52	-0.43	-0.32	-0.35	-0.32	0.37	0.41	0.48	0.41
p-value	0.09	0.01	0.02	0.02	0.08	0.00	0.02	0.08	0.06	0.08	0.05	0.02	0.01	0.02

Starchy Vegetables	Alistipes	Lachnospiraceae	Oscillospira	Dialister
ρ	0.07	0.31	-0.41	-0.34
p-value	0.70	0.09	0.03	0.06

Juice	Rikenellaceae	Lachnospiraceae	Butyrivibrio	Faecalibacterium	Mogibacteriaceae
ρ	0.39	-0.31	-0.36	-0.41	0.31
p-value	0.03	0.09	0.05	0.02	0.09

Sweetened Beverages	Alistipes	Oscillospira	Dialister	Phascolarctobacterium	Catenibacterium	RF32
ρ	-0.43	0.42	0.33	-0.38	0.41	-0.36
p-value	0.02	0.02	0.07	0.04	0.02	0.05

Grains	Coriobacteriaceae	Adlercreutzia	Collinsella	Rikenellaceae	Barnesiellaceae	Lactococcus	Streptococcus	Clostridiaceae_family	Sarcina	Lachnospiraceae	Roseburia	Ruminococcaceae	Succiniclasticum	Campylobacter	Actinobacteria	Firmicutes
ρ	0.38	0.50	0.57	0.31	0.42	0.33	0.35	0.31	-0.41	0.37	0.41	0.56	0.43	0.48	0.34	0.50

Supplemental Table 5.1 (cont.)

p-value	0.04	0.01	0.00	0.10	0.02	0.08	0.06	0.10	0.02	0.04	0.02	0.00	0.02	0.01	0.06	0.01

Refined Crabohydrates	Slackia	Rikenellaceae	Anaerotruncus	Oscillospira	Dialister	Catenibacterium
ρ	-0.51	0.42	0.34	0.31	0.31	0.40
p-value	0.00	0.02	0.07	0.09	0.09	0.03

Fried Food	Bifidobacterium	Bacteroides	Alistipes	Clostridiaceae_family	twod06	SMB53	Peptostreptococcaceae	Erysipelotrichaceae	Catenibacterium	RF32	Bilophila	Actinobacteria	Bacteroidetes	Firmicutes
ρ	-0.31	0.33	-0.34	-0.55	-0.42	-0.39	-0.35	-0.35	0.42	-0.39	0.31	-0.32	0.41	-0.36
p-value	0.09	0.07	0.07	0.00	0.02	0.03	0.06	0.06	0.02	0.04	0.09	0.08	0.02	0.05

Protein	Eggerthella	Lactobacillus	Pseudoramibacter_Eubacterium	Coprococcus	Oscillospira	Phascolarctobacterium	Enterobacteriaceae
ρ	-0.32	-0.31	-0.40	0.39	-0.43	0.37	0.32
p-value	0.09	0.09	0.03	0.03	0.02	0.04	0.08

Dairy	Pseudoramibacter_Eubacterium	Anaerotruncus	Succiniclasticum
ρ	-0.31	0.35	0.32
p-value	0.09	0.05	0.08

Snacks	Eggerthella	Bacteroides	Alistipes	Butyricimonas	Odoribacter	Clostridium	SMB53	Roseburia	Oscillospira	Dialister	Phascolarctobacterium	Erysipelotrichaceae_Eubacterium	RF32	Sutterella	Enterobacteriaceae	RF39	Tenericutes
ρ	0.43	0.33	-0.49	-0.36	-0.33	-0.33	-0.34	-0.37	0.59	0.52	-0.60	0.37	-0.35	0.32	-0.55	0.46	0.46
p-value	0.02	0.08	0.01	0.05	0.07	0.07	0.07	0.04	0.00	0.00	0.00	0.04	0.06	0.08	0.00	0.01	0.01

Supplemental Table 5.1 (cont.)

Sweets	Bacteroides	Alistipes	Enterococcus	Clostridiaceae_family	SMB53	Roseburia	Peptostreptococcaceae	Oscillospira	Dialister	Holdemania	RF32	Enterobacteriaceae	Actinobacteria	Bacteroidetes
ρ	0.34	-0.36	-0.34	-0.34	-0.45	-0.33	-0.42	0.42	0.37	-0.36	-0.38	-0.34	-0.31	0.35
p-value	0.07	0.05	0.06	0.07	0.01	0.07	0.02	0.02	0.05	0.05	0.04	0.07	0.09	0.06

Kids Meal	Coriobacteriaceae	Bacteroides	Alistipes	Clostridiaceae_family	SMB53	Blautia	Peptostreptococcaceae	Erysipelotrichaceae	Catenibacterium	Holdemania	Actinobacteria	Bacteroidetes
ρ	-0.44	0.47	-0.63	-0.62	-0.54	-0.38	-0.48	-0.34	0.34	-0.40	-0.38	0.49
p-value	0.02	0.01	0.00	0.00	0.00	0.04	0.01	0.06	0.07	0.03	0.04	0.01

Fish	Enterococcus	twod06	Pseudoramibacter_Eubacterium	Lachnospiraceae_Ruminococcus	Peptococcus	Erysipelotrichaceae_Eubacterium	Enterobacteriaceae	Akkermansia	Bacteroidetes	Verrucomicrobia
ρ	0.35	-0.33	-0.35	-0.39	-0.37	-0.34	0.32	-0.31	0.32	-0.31
p-value	0.05	0.07	0.06	0.03	0.04	0.06	0.09	0.09	0.09	0.09

Condiments	Adlercreutzia	Streptococcus	Dialister
ρ	0.41	0.34	0.31
p-value	0.02	0.07	0.10

Supplemental Table 5.2 Correlation between VFA and Food and nutrient intake for all groups at baseline

a. ASD group

Food/Nutrient	VFA	ρ	p-value
Dairy	Acetate	0.33	0.09
Sweetened Beverages	Isobutyrate	0.35	0.08
Starchy Vegetables	Isovalerate	-0.35	0.08
Grains	Isovalerate	-0.36	0.07
Starchy Foods	Valerate	0.38	0.06
Energy	Isobutyrate	-0.43	0.03
Energy	Isovalerate	-0.41	0.04
Total Fat	Isobutyrate	-0.37	0.06
Total Fat	Isovalerate	-0.35	0.08
Total Carbohydrate	Isobutyrate	-0.46	0.02
Total Carbohydrate	Isovalerate	-0.44	0.03
Total Dietary Fiber	Isobutyrate	-0.43	0.03
Total Dietary Fiber	Isovalerate	-0.41	0.04
Total Dietary Fiber	Valerate	-0.34	0.09
Soluble Dietary Fiber	Isobutyrate	-0.46	0.02
Soluble Dietary Fiber	Isovalerate	-0.42	0.03
Pectins	Isobutyrate	-0.39	0.05
Pectins	Isovalerate	-0.38	0.06
Pectins	Valerate	-0.48	0.01

b. CONT group

Food/Nutrient	VFA	ρ	p-value
Sweetened Beverages	Acetate	0.31	0.09
Sweetened Beverages	Butyrate	0.45	0.01
Sweetened Beverages	Isobutyrate	0.42	0.02
Sweetened Beverages	Isovalerate	0.41	0.02
Sweetened Beverages	Valerate	0.59	0.0005
Snacks	Acetate	0.33	0.07
Snacks	Propionate	0.32	0.07
Snacks	Butyrate	0.3	0.09
Snacks	Isobutyrate	0.34	0.06
Snacks	Isovalerate	0.34	0.06
Snacks	Valerate	0.37	0.04
Dairy	Propionate	0.38	0.03
Kid's Meals	Propionate	0.3	0.09
Fruit	Isobutyrate	0.41	0.02
Fruit	Isovalerate	0.43	0.01
Starchy foods	Isobutyrate	0.31	0.08
Starchy foods	Isovalerate	0.33	0.09
Starchy foods	Valerate	0.38	0.03

Supplemental Table 5.2 (cont.)

Food/Nutrient	VFA	ρ	p-value
Refined Carbohydrates	Isovalerate	0.34	0.06
Refined Carbohydrates	Isobutyrate	0.33	0.07
Condiments	Acetate	0.31	0.08
Condiments	Butyrate	0.41	0.02
Condiments	Isobutyrate	0.41	0.02
Condiments	Isovalerate	0.39	0.02
Condiments	Valerate	0.44	0.01
Total Dietary Fiber	Valerate	-0.34	0.07
Soluble Dietary Fiber	Valerate	-0.35	0.06
Insoluble Dietary Fiber	Valerate	-0.34	0.07
Pectins	Valerate	-0.33	0.07
Refined Grains	Acetate	0.35	0.06
Refined Grains	Butyrate	0.35	0.06
Refined Grains	Isobutyrate	0.35	0.06
Refined Grains	Isovalerate	0.32	0.09

Correlations were analyzes using Spearman (non-normal) and Pearson (normal) correlation coefficient

CHAPTER 6

Longitudinal Dynamics in Fecal Microbiota Composition in Children with ASD and Association with Dietary Patterns

Abstract

Background/Aims: The gastrointestinal (GI) microbiota undergoes changes throughout time and variability in composition is observed within the course of a day or over weeks. Environmental factors such as diet have been shown to influence long-term microbial stability and microbial stability was associated with disease states. Microbial dysbiosis is often observed in children with Autism Spectrum Disorder (ASD); however, little is known about temporal stability as well as the impact of dietary patterns on the microbial stability in children with ASD. Likewise, whether microbial variability is related to ASD symptoms is unknown.

Methods: The temporal variability in stool microbiota composition in relation to dietary habits in children with ASD (ASD, n=26) and unaffected controls (CONT, n=32) aged 2-7 years was investigated. Fecal samples were collected at baseline, 6-weeks and 6-months. Bacterial composition was assessed using 16S rRNA sequencing. Volatile fatty acid (VFA) concentrations were analyzed using gas chromatography. Nutrient intake was assessed using a 3-day food diary and dietary patterns were empirically derived from a food frequency questionnaire. Social deficit scores (SOCDEF) were assessed using the Pervasive Developmental Disorder Behavior Inventory-Screening Version (PDDBI-SV). Gastrointestinal (GI) symptoms were assess using the GI severity index.

Results: Overall, temporal variability in microbial structure and membership did not differ between the groups. However, fluctuations in different microbial taxa and microbial metabolites were observed in the two groups. Specifically, abundances of Clostridiaceae, Streptophyta,

Eggerthella, *Clostridium*, *Roseburia* and *Dialister* showed significant variations in ASD over the 6-month study period. In CONT, changes in abundances of Enterobacteriaceae, Clostridiaceae, and *Enterococcus* were observed. Regarding VFA, concentration of all SCFA (acetate, propionate, butyrate) and BCFA (isobutyrate, isovalerate and valerate) decreased in children with ASD over time, whereas only acetate concentration increased in CONT children over a 6-month period. Variability in community membership measured by median unweighted UniFrac distance negatively correlated with median SOCDEF T-scores. Additionally, different bacterial taxa contributed to microbial stability in the two groups. Clostridiales, Ruminococcaceae, *Lactococcus*, *Turicibacter*, *Dorea*, and *Phascolarctobacterium* were components of a more stable microbiota community in children with ASD whereas Barnesiellaceae, *Adlercreutia*, *Faecalibacterium*, *Sutterella* and *Bilophila* were components of the stable microbiota in CONT children. Lastly, dietary measures were associated with long-term stability of the microbiota in both groups. In children with ASD, a dietary pattern characterized by vegetables, starchy vegetables, legumes, nuts and seeds, fruit, grains, juice and dairy was associated with changes in species diversity (Shannon Index) and richness (Chao 1 Index), abundance of Erysipelotricaceae, *Clostridium*, *Oscillospira* and *Dorea* as well as concentrations of propionate, butyrate, isobutyrate and isovalerate. A dietary pattern characterized by intakes of fried foods, kid's meals, protein foods, condiments, snacks and starchy foods was associated with variations in microbiota structure, abundance of Cyanobacteria, Erysipelotrichaceae, *Clostridium*, and *Oscillospira* as well as changes in the concentration of all VFAs. No changes in SOCDEF and GI severity scores were observed over the 6-month study period.

Conclusion: Microbiota composition varies over time in children with ASD and might be related to dietary intake and social defict scores. Future studies investigating the physiological effect of

the changes in specific microbial taxa as well as metabolites are needed to delineate the impact on ASD symptomology.

6.1 Introduction

During the first year of life, the gastrointestinal (GI) microbiota undergoes rapid changes (Koenig et al., 2011). Although previous studies have suggested that the GI microbiota becomes relatively stable and resembles that of an adult at 3 years of age (Bergström et al., 2014; Palmer et al., 2007; Yatsunenko et al., 2012, other studies suggest that the GI microbiota might have a more prolonged development, lasting into pre-adolescence (Hollister et al., 2015). One study reported 70% stability in microbiota composition over the 18-months in children aged 2-18 years, with Bacteroidetes showing the highest compositional stability followed by Proteobacteria (de Meij et al., 2016). Other studies have shown that even in adults the microbiota composition can be highly variable and changes over the course of a day (Kaczmarek et al., 2017) or weeks (Caporaso et al., 2011; Flores et al., 2014) have been reported. Thereby, bacteria that are lower abundance might be more transient and prone to temporal variations than predominant phyla of the bacterial core (Durbán et al., 2012; Vanhoutte et al., 2004; Caporaso et al., 2011).

Increasing reports show that the GI microbiota differs between children with Autism Spectrum Disorder (ASD) and unaffected controls; however, longer term variability in the ASD population has not previously been studied. Understanding the long-term variability of the GI microbiota is important as resistance to environmental stressors and greater resistance to pathogen invasion and a rapid return to a baseline state are key features of a healthy microbiome (Bäckhed et al., 2012; Ley et al., 2008). Previous studies have linked a greater microbiota instability to certain disease states (e.g., Crohn's disease) (Mättö et al., 2013; Maukonen et al.,

2006; Scanlan et al., 2006). Although extreme shifts in the intake of dietary macronutrient and fiber are associated with short-term changes in the GI microbiota, most studies have found that these shifts are reversible and that the GI microbiota returns to a baseline composition (David et al., 2014, Li et al., 2017). We have previously identified a dietary pattern that was characterized by higher intakes of vegetables and lower intakes of snacks, sweets and dairy food to be associated with a greater microbial stability over a 6-month period, showing a direct relationship between dietary patterns and temporal variability of the microbiota in children (Berding et al., 2018).

Given the new evidence showing that specific microbes could contribute to some symptoms of ASD, identifying long-term dietary patterns that could potentially promote the development of a more beneficial microbiota composition could be important advances in the understanding of the microbiota-brain connection in ASD. We hypothesize that children with ASD will have more variability in their microbiota compared to unaffected controls. We further hypothesize that in children with ASD a dietary pattern characterized by higher intakes of healthy foods such as fruits, vegetables and grains will be associated with a more stable microbiota composition. Lastly, more microbial variability will be associated with more severe ASD symptoms.

6.2 Materials and Methods

Study Design, Participants, Fecal Sample Collection and analysis, 3-day Food Diary, and Assessment of ASD Symptoms

The study design, participant recruitment and sample collection details are described in Chapter 4. Additional samples were collected 6-weeks and 6-months after baseline sample

collection. Fecal samples for microbiota and VFA analysis were collected and analyzed as described in Chapter 4. Likewise, nutrient intake measured by 3-day food records and the Youth and Adolescence Food frequency questionnaire as well as information on ASD and GI symptoms were collected as described in Chapter 5.

Statistics

All data were analyzed using SAS 9.4 (SAS Institute, Cary, NC). Baseline dietary patterns were derived as previously described (Chapter 5).

The 16S rRNA sequences were processed and analyzed using QIIME 1.9.1 bioinformatics software as previously described (Chapter 4). Sequences were rarefied to a sampling depth of 36,565 sequences per sample for subsequent analysis.

Variability in diversity measures (α- and β-diversity) was assessed for participants who provided all three sample (ASD n=22; CONT n=29). Variability of α-diversity (within an individual over time) was assessed by calculating the coefficient of variation (CV=standard deviation/mean) for Chao 1 index, observed OTUs and Shannon index (Shade et al., 2013). Higher values indicated a more variable community. The median weighted and unweighted UniFrac distances for each individual over time were calculated to determine the variability in community composition over time (Flores et al., 2014). Higher median values correspond to more variability whereas lower median values are indicative of a more stable microbial community.

For both groups, the study population was divided into stability classes (falling above or below the median based on median weighted UniFrac distances) in order to determine which bacteria could contribute to the stability or instability of the overall composition (Flores et al.,

2014; Galloway- Peña et al., 2017). Children falling above the median were denoted as less stable whereas children below the median were denoted as more stable.

In order to determine the impact of factors on changes in the microbiota composition, microbiota variability was analyzed based on baseline dietary patterns (as described in Chapter 5), introduction of specialty diets and severity of social deficit symptoms. Regarding dietary patterns, children were dichotomized as falling above or below the median in each dietary pattern. Additionally, children in the ASD group were dichotomized by introducing specialty diets over the 6-month period and were grouped according to baseline severity of ASD symptoms (i.e., moderate or severe). Differences in outcome measures were analyzed using mixed models including participant as a repeated effect. Model fit was assessed using the Chi-square-to-df ratio. Values <2 were indicative of an appropriate model fit (Kaczmarek et al., 2017). Factors known to influence the microbiota composition including age, gender, BMI, season of sample collection, introduction of medication or probiotics and specialty diet as well as food groups and nutrients that changed significantly over the 6-month period were included as co-variates. Spearman correlation was used to investigate relationships between variability and ASD symptoms. Data are expressed as median (IQR) or mean ± SD. Level of significance was set at $p \leq 0.05$ and $p \leq 0.10$ was considered a trend.

6.3 Results

Enrollment and Attrition Rate of Study Population

The enrollment and attrition rate of study participants is shown in **Table 6.1**. In the CONT group, all subjects provided samples at the 6-week time point and 29 subjects provided samples at the 6-month time point. In the ASD group, one subject failed to provide a stool

sample at the 6-week time point and 4 subjects failed to provide samples at the 6-month time point. Thus, for the ASD group, 25 samples were collected at the 6-week time point and 22 samples were collected at the 6-month time point. Each analysis described below was also conducted only including children who completed all three sample collections (data not shown). Statistically, these analyses did not differ from the analyses including the full cohort; thus, the data including all subjects is reported herein.

Introduction of Medication, Specialty Diets or Probiotics

Introduction of medication, specialty diets and probiotics by group are shown in **Table 6.2**. In ASD, one child took medication within a month prior to the 6-week time point and three children took medication prior to the 6-month time point. These medications included Clonidine, Conerta (stimulant), and Namenda (cognitive enhancing medicine). In CONT, three children took medication within a month prior to the 6-week sample collection and one child prior to the 6-month sample collection. These medications included cold relief medication and other antibiotics. Regarding specialty diets, no children in the CONT group started a specialty diets. In the ASD group four children were following a specialty diet at the 6-week sample collection and seven children at 6-month time point. These diets included the gluten-free/casein-free diet and other exclusions diets (i.e., decrease sugar alcohols and fructose). Probiotics were not introduced in either of the groups.

GI Symptoms, Stool Consistency and ASD Symptoms

GI symptoms (**Figure 6.1**) and stool consistency (**Table 6.3**) as measured by the Bristol stool chart did not change in either group over the 6-month time period. Additionally, no significant changes on SOCDEF T-scores were observed in the ASD group (**Figure 6.2**).

Nutrient Intake from Baseline to 6-Month Post-Baseline

Nutrient intake from the 3-day food records were analyzed for changes over the 6-month period to determine the potential impact on the temporal variability of the GI microbiota. Nutrients that changed significantly over the 6-month period are shown in **Table 6.4 a**.

In the ASD group, a decrease in intake of total carbohydrate (p=0.04), vitamin A (p=0.02), vitamin E (p=0.04), and magnesium (p=0.02) was observed. Additionally, intakes of linoleic acid (p=0.02), total sugars (p=0.03) and added sugars (p=0.01) changed during the 6-month period with the highest intakes reported at the 6-week sample collection. In the CONT group, no significant changes in nutrients were observed.

Food Group Intake from Baseline to 6-Month Post-Baseline

Regarding intake of food groups, no changes were observed in the ASD group. In the CONT group, intake of starchy vegetables (p=0.01) and protein foods (p=0.0001) increased from baseline to 6-week and 6-month post-baseline with the highest intake at the 6-week time point. Intakes of all food groups for both groups are shown in **Table 6.4b**.

Differences in Temporal Microbial Diversity Between the Groups

Temporal variability within each group quantified by the coefficient of variation (CV) did not differ between the groups (**Figure 6.3**). Likewise, temporal variability in community

membership (unweighted UniFrac) and structure (weighted UniFrac) did not differ between the groups (**Figure 6.4**).

Temporal Microbial Diversity and Composition within Each Group

Abundance of individual bacterial taxa that differed significantly between the three time points in both groups can be found in **Table 6.5.** There were no differences in measures of species richness (Chao 1 Index and observed OTUs) and diversity (Shannon and Simpson Index) or in bacterial abundance at the phyla level over 6-month period in either group. Additionally, diversity between time points measured by β-diversity did not differ in either group.

Analyzing changes in specific microbial taxa showed that different bacteria changed over a 6-month period in each group. In ASD children, abundance of *Streptophyta* (p=0.07) and *Dialister* (p=0.07) increased whereas abundances of Clostridiaceae (p=0.01), *Clostridium* (p=0.003), and *Roseburia* (p=0.02), decreased over the 6-month study period, with the highest abundance of Clostridiaceae and *Roseburia* being observed at the 6-week time point.

In CONT children, an increase in the abundance of Enterobacteriaceae (p=0.01) and *Enterococcus* (p=0.08) was observed over the 6-month study period.

Association between Microbial Variability and ASD Symptoms

Interestingly, in children with ASD variability based on community membership (unweighted UniFrac) tended to be negatively correlated (ρ=-0.38; p=0.07) with median SOCDEF scores over the 6-month period (**Figure 6.5**). The diversity within each child with ASD (based on CV for α-diversity measures) was not associated with SOCDEF scores.

VFA Concentrations from Baseline to 6-Month Post-Baseline

Concentration of all VFAs at baseline and 6-month post-baseline can be found in **Figure 6.6a** and **6.6b**. In ASD, a statistically significant decrease in the concentration of acetate (p=0.01), propionate (p=0.05), butyrate (p=0.02) and isobutyrate (p=0.01) and a trend for a decrease in isovalerate (p=0.05) and valerate (p=0.09) concentrations was observed. In CONT, the concentration of acetate (p=0.07) tended to increase over the 6-month study period.

mmdA and BCoAT Gene Expression

The ratios of mmDA and BCoAT to total 16S rRNA at baseline, 6-week and 6-month post-baseline are shown in **Figure 6.6 c and d**. There was no statistically significant difference in the expression of mmDA and BCoAT in either group.

Bacterial Taxa Contributing to Stability of Microbial Structure

In order to determine whether specific bacterial taxa could contribute to a more or less stable microbial structure, differences in the average relative abundance of bacterial taxa between children dichotomized by stability category (more stable vs. less stable) were assessed.

ASD group: **Figures 6.7 (a-l)** represent all bacterial taxa that were significantly different between stability categories in children with ASD. Coriobacteriaceae (p=0.06) was more abundant in children with ASD categorized as having a less stable microbiota based on median weighted UniFrac distance. On the other hand, Clostridiales (p=0.008), Ruminococcaceae (p=0.05), *Lactococcus* (p=0.002), *Turicibacter* (p=0.01), *Dorea* (p=0.008) and

Phascolarctobacterium (p=0.005) were more abundant on average in children with ASD whose microbiota structure is temporally more stable over a 6-month period. Measures of bacterial richness and diversity did not differ between children categorized by having a more or less stable microbiota (data not shown). SCODEF T and GI symptoms severity scores did not change in either stability category.

CONT group: Diversity and richness measures as well as bacterial taxa that significantly differed based on stability category in CONT children are shown in **Table 6.6**. Children with a more stable microbial community had higher species richness based on Chao1 index (p=0.07) and higher species diversity as measured by the Shannon index (p=0.06) compared to children with a less stable microbiota. Verrucomicrobia (p=0.02), Enterobacteriaceae (p=0.09) and *Akkermansia* (p=0.002) were more abundant in CONT children with a more unstable microbiota. On the other hand, Barnesiellaceae (p=0.06), *Adlercreutzia* (p=0.005), *Faecalibacterium* (p=0.02), *Sutterella* (p=0.002), and *Bilophila* (p=0.06) were more abundant in CONT children with a more stable microbiota community over the 6-month study period.

Dietary Factors Contributing to Microbial Stability

In order to determine whether specific dietary factors could contribute to a more stable microbial profile, differences in the average intake of food groups and nutrients based on stability class were investigated.

ASD group: **Table 6.7** shows the dietary intake that could contribute to microbial stability in children with ASD. Children with ASD that had a more stable microbial community

had higher intakes of fish (p=0.03) and total protein (p=0.04) whereas children with ASD categorized as having lower microbial stability had higher intakes of snacks (p=0.07) and condiments (p=0.03)

CONT group: Dietary intake based on the category of microbial stability in CONT children is shown in **Table 6.6**. CONT children with a more stable microbiota had higher intakes of whole grains (p=0.008), vegetables (p=0.05), sweetened beverages (p=0.08), fish (p=0.01) and condiments (p=0.03), whereas children with a less stable microbiota had higher intakes of kid's meals (p=0.009).

Microbiota Composition and VFA Concentration Based on Introduction of Specialty Diets in Children with ASD

In order to investigate whether the introduction of a specialty diet impacts microbiota dynamics, the microbiota composition over the 6-months period was analyzed in the context of specialty diets. Overall microbial structure and membership (median weighted and unweighted UniFrac distances) as well as within sample diversity (CV of α-diversity matrices) was not impacted by the introduction of specialty diets.

Regarding nutrient intake, children with ASD that were following a specialty diet (SPEC) at the 6-week and 6-month time-points had decreased intakes of vegetables (p=0.09) and increased intakes of fish (p=0.06) at those time points. Additionally, decreased intakes of added sugars (p=0.02) were observed (**Table 6.8**). Children with ASD who did not start following a specialty diet (NO-SPEC) during the duration of the study had decreases in intake of vitamin E (p=0.04) and sodium (p=0.06) as well as increased intake of folate (p=0.08) (**Table 6.8**).

A summary of changes in bacteria and VFA concentrations based on specialty diet can be found in **Table 6.9**. Children in the SPEC group had significant changes in abundance of Actinobacteria (p=0.04), Clostridiaceae (p=0.02), and *Bifidobacterium* (p=0.005) whereas children in the NO-SPEC group had significant changes in the abundance of Bacteroidetes (p=0.06) and *Clostridium* (p=0.002).

Lastly, NO-SPEC children had significant decreases in in the concentration of propionate (p= 0.01), butyrate (p=0.002) and isobutyrate (p=0.05). On the other hand, SPEC children had significant decreases in acetate concentrations (p=0.04).

There were no changes in SOCDEF T- or GI symptoms scores in either group over the 6-month period.

Nutrient and Food Group Intake, Microbiota Stability and VFA Concentrations over 6-Month Period Based on Baseline Dietary Patterns

Changes in intake of nutrients and food groups as well as microbial stability and changes in VFA concentrations over the 6-month study period were investigated based on dietary patterns for both study groups. Description of dietary patterns can be found in **Table 5.4 (pp. 195)**. Briefly, in children with ASD, Dietary Pattern 1 (DP1-ASD) was characterized by an intake of vegetables, legumes, nuts and seeds, fruit, starchy vegetables, grains, juice and dairy. Dietary Pattern 2 (DP2-ASD) was characterized by an intake of fried foods, kid's meals, condiments, protein foods, snacks and starchy foods. In the CONT group, Dietary Pattern 1 (DP1-CONT) was characterized by intakes of sweets, kid's meals, fried foods, snacks, starchy foods, dairy and sweetened beverages, whereas Dietary Pattern 2 (DP2-CONT) was characterized by intakes of fish, vegetables, protein foods, fruit, and juice.

ASD – Dietary pattern 1: There was no difference in temporal variability of community membership (unweighted UniFrac) or structure (weighted UniFrac) based on DP1-ASD (**Figure 6.8a**). Likewise, temporal variability in α-diversity did not differ based on DP1-ASD (**Table 6.10**).

In DP1-ASD, intake of servings per day of fruit (p=0.04), vegetables (p=0.06) and juice (p=0.07) as well as intake of total carbohydrates (p=0.09) and added sugars (p=0.03) tended to decrease over the 6-month study period in children above the median (**Table 6.11a**). Regarding the microbiota composition, an increase in the relative abundance of Erysipelotrichaceae (p=0.05) and a decrease in the abundance of *Clostridium* (p=0.03) was observed. (**Table 6.12a**). VFA concentrations did not change in children above the median in DP1-ASD (**Figure 6.9a**).

More changes in nutrient and food group intake and microbiota composition were observed in children below the median in DP1-ASD. An increase in servings per day of legumes, nuts and seeds (p=0.01), juice (p=0.09) and refined carbohydrates (p=0.001) as well as a decrease in vitamin A (p=0.01), folate (p=0.05), phosphorus (p=0.07) and refined grains (p=0.07) was observed in children below the median in DP1-ASD (**Table 6.11a**). Regarding the microbiota composition, species diversity based on Shannon index (p<0.0001) as well as species richness based on Chao1 Index (p=0.04) decreased in children below the median in DP1-ASD. Additionally, abundance of *Clostridium* (p=0.01), *Oscillospira* (p=0.002) and *Dorea* (p=0.05) decreased over the 6-month study period in children below the median in DP1-ASD (**Table 6.12a**). Lastly, concentrations of propionate (p=0.08), butyrate (p=0.02), isobutyrate (p=0.09) and isovalerate (p=0.06) decreased in children below the median in DP1-ASD (**Figure 6.9b**).

ASD – Dietary pattern 2: Community structure variability (median weighted UniFrac) tended (p=0.07) to differ based on DP2-ASD. Children above the median in DP2-ASD tended to

have higher variability (higher median values) compared to children below the median (**Figure 6.8b**). There was no difference based on DP2-ASD in median unweighted UniFrac or temporal variability of α-diversity (**Table 6.10**).

In DP2-ASD, children with ASD falling above the median had decreased intakes of protein foods (p=0.06), vitamin A (p=0.01), vitamin B_{12} (p=0.07) and sodium (p=0.04) over the 6-month study period (**Table 6.11b**). Regarding the microbiota composition, a decrease in the abundance of Erysipelotrichaceae (p=0.07), *Clostridium* (p=0.005) and *Oscillospira* (p=0.008) was observed (**Table 6.12a**). Additionally, concentrations of propionate (p=0.08), butyrate (p=0.02), isobutyrate (p=0.02), isovalerate (p=0.02) and valerate (p=0.04) decreased in children above the median in DP2-ASD (**Figure 6.9c**).

Children falling below the median in DP2-ASD had increased intakes of refined carbohydrates (p=0.04) and decreased intakes of vitamin E (p=0.05) and added sugars (p=0.06) (**Table 6.11b**). The microbial profile was characterized by a decrease in Cyanobacteria (p=0.07), and *Clostridium* (p=0.06) (**Table 6.12a**). Acetate (p=0.04) and propionate (p=0.02) concentrations decreased significantly in children falling below the median in DP2-ASD (**Figure 6.9d**).

SOCDEF and total GI severity scores did not significantly change in either dietary pattern in children with ASD.

CONT – Dietary pattern 1: There were no differences based on median weighted or unweighted UniFrac or in α-diversity based on either dietary pattern in unaffected controls (data not shown).

Children above the median in DP1-CONT had an increased intake of servings per day of protein foods (p=0.03) as well as total protein (p=0.09), vitamin B_{12} (p=0.06), and sodium

(p=0.07) and a decreased intake of servings per day of snacks (p=0.08) and added sugars (p=0.004). Additionally, intakes of manganese (p=0.02) and whole grains (p=0.06) increased from baseline to 6-weeks, but then decreased at the 6-month time point and were lower compared to baseline intakes (**Table 6.13a**). The microbial profile of children above the median in DP1-CONT was characterized by a decrease of *Parabacteroides* (p=0.03) (**Table 6.14a**).

Nutritionally, intakes of servings per day of fruit (p=0.03) and grains (p=0.07) increased while intake of servings per day of fried food (p=0.04) decreased in children below the median in DP1-CONT. Likewise, intake of vitamin D (p=0.02), riboflavin (p=0.03), vitamin B_{12} (p=0.08) and selenium (p=0.06) decreased over the 6-month study period in children below the median in DP1-CONT (**Table 6.13a**). Furthermore, a decrease in *Butyrivibrio* (p=0.03) and *Veilonella* (p=0.04) was observed in children below the median in DP1-CONT (**Table 6.14a**).

CONT – Dietary pattern 2: Intake of vitamin K (p=0.02), vitamin C (p=0.006) and added sugars (p=0.09) decreased in children above the median in DP2-CONT, with the intake of added sugars being lowest at the 6-week time point (**Table 6.13b**). Children above the median in DP2-CONT showed a decrease in relative abundance of Proteobacteria (p=0.07) and *Slackia* (p<0.001) (**Table 6.14b**).

Regarding food group intake, increased consumption of servings per day of fruit (p=0.01), refined carbohydrates (p=0.06) and protein foods (p=0.004) while decreased consumption of legumes, nuts and seeds (p=0.002) was observed. Furthermore, an increased consumption of total fat (p=0.02), SFA (p=0.05) and vitamin K (p=0.07) as well as a decreased consumption of whole grains (p=0.006) was detected in children below the median in DP2-

CONT (**Table 6.13b**). Bacterial abundances did not significantly change over the 6-month period in children below the median in DP2-CONT (**Table 6.14b**).

VFA concentration or GI severity index did not change in either dietary pattern in the CONT group (data not shown).

Temporal Microbiota Composition and Diversity Based on Severity of Social Deficit Symptoms

In order to analyze whether the longitudinal stability of the GI microbiota is associated with social deficit severity, children with ASD were dichotomized based on baseline level of severity of social deficit scores (i.e., mild, moderate or severe). Only two children fell into the mild severity category of SOCDEF scores; thus, longitudinal dynamics for the mild category was not included in this analysis.

There was no difference in the amount of children starting a specialty diets based on symptoms severity. Four children in the moderate category and two children with severe social deficits started a specialty diet. Regarding medication use, one child with moderate and two children with severe social deficits introduced medication over the 6-month study period.

The results for changes in microbiota, VFA concentration and nutrition can be found in **Tables 6.15** and **6.16**. There were no differences in microbiota composition over the 6-month period in α-diversity or at the phyla level in either severity group.

Regarding the nutritional intake, children with ASD and moderate levels of social deficit had a decrease in intake of vitamin A ($p=0.04$), vitamin B_{12} ($p=0.06$), sodium ($p=0.07$) and added sugars ($p=0.07$). In children with moderate levels of social deficits, an increase in *Staphylococcus* ($p=0.07$) and decrease in *Clostridium* ($p=0.006$), *Sarcina* ($p=0.02$) and *Haemphilus* ($p=0.05$) was observed. Additionally, concentrations of propionate ($p=0.02$),

butyrate (p=0.01), isobutyrate (p=0.007) and isovalerate (p=0.03) decreased over the 6-month study period.

There were no changes in food group and nutrient intake in children with ASD and severe levels of social deficits. In children with severe levels of social deficits, abundances of Clostridiaceae (p=0.09) and *Methanobrevibacter* (p=0.01) increased from baseline to 6-weeks and decreased from 6-weeks to 6-months post-baseline. Regarding VFA concentrations, acetate (p=0.03), propionate (p=0.01), and butyrate (p=0.003) levels decreased over the 6-month study period.

6.4 Discussion

Microbial stability is defined as the "ability to respond to perturbations by resisting change and returning to the original state" (Holling, 1973). Thereby, higher microbial stability is associated with a healthier microbiota due to the ability to maintain bacterial function and to resist environmental stressors (Bäckhed et al., 2012; Coyte et al., 2015). Short-term intra-individual variability during weeks or months and over the course of a day have been reported (Caporaso et al., 2011; Flores et al., 2014; Kaczmarek et al., 2017), with different body sites showing various degrees of variation (Flores et al., 2014). Some studies suggest that a stable individual microbial core containing predominant microbial taxa exists (Jalanka-Tuovinen et al., 2011; Li et al., 2013; Jalanka, 2014; Lozupone et al., 2012). Thereby, microbial diversity was a predictor of a more stable microbiota (Flores et al., 2014). Additionally, a stable microbiota with high levels of commensal bacteria could be important in protecting against infection by pathogens (Galloway-Peña et al., 2017) whereas low abundance members may contribute more significantly to the variation in the microbiome (Li et al., 2013; Faith et al., 2013). One study

found that over a 5-year period 60% of sequences were persistently present and species within the phyla Bacteroidetes and Actinobacteria were the most stable (Faith et al., 2013). Furthermore, Clostridiaceae and Lactobacillaceae seemed to be more abundant in individuals with a high microbial variability, whereas Bacteroidaceae was most abundant in stable individuals (Flores et al., 2014). Likewise, the degree of variation in the microbiome might be specific to each individual, meaning that microbial taxa that are stable in one individual might undergo more variation in another indivudual (Jalanka, 2014).

Understanding microbial dynamics is important to define stable states of the microbiota and to inform potential interventions that could promote health by stabilizing the microbial community (Lozupone et al., 2012; Flores et al., 2014). Perturbations of the microbial equilibrium could potentially lead to an unhealthy host status (Moya & Ferrer, 2016; Lozupone et al., 2012) as microbial instability has been linked to certain diseases (Li et al., 2013; Mättö et al., 2013). For example, a greater instability was found in patients with IBS and Crohn's disease (Mättö et al., 2013; Maukonen et al., 2006; Scanlan et al., 2006). Increasing evidence supports microbial dysbiosis in children with ASD and some studies suggest that specific bacterial taxa might be associated with some symptoms of ASD (Tomova et al., 2015; Kang et al., 2013). However, little is known about the microbial stability in children with ASD and whether it could be linked to on symptom severity. Additionally, various environmental factors such as host health (Claesson et al., 2012), genetics (Khachatryan et al., 2008), age and diet (Berding et al., 2018) are known to influence the abundance of bacterial taxa. Dramatic changes in short-term dietary intake can rapidly modify the composition and function of the GI microbiota (David et al., 2014). On the other hand, habitual dietary patterns have been shown to influence the stability GI microbiota composition (Berding et al., 2018). Achieving a balanced dietary intake can often

be challenging in children with ASD (Ledford & Gast, 2006) and dietary interventions (i.e., gluten free/casein free diet) are commonly used by parents in an effort to alleviate some symptoms of ASD (Marí-Bauset et al., 2016). However, how habitual dietary patterns are potentiality correlated with the microbiota stability in children with ASD is unknown.

To fill these gaps, three fecal samples as well as information on dietary habits from children with ASD and unaffected controls were collected over a 6-month period. Microbial stability in relation to dietary patterns, use of specialty diets and severity of ASD symptoms was investigated to further elucidate the relationship between diet, microbiota and symptoms of ASD. Changes in the microbiota composition of children with ASD and unaffected control children were observed, with different bacterial taxa contributing to a stable microbiota composition in both groups. Additionally, in children with ASD microbial stability was associated with dietary interventions, level of ASD symptom severity and baseline dietary pattern and food intake. However, no direct association with severity of social deficits was observed. Thus, this study supports the hypothesis that long-term eating patterns in children with ASD have different effects on the temporal variability of the microbiota, but additional research is needed to further delineate the potential impact on symptoms of ASD.

Recent studies have linked microbial stability to certain diseases and potentially mood (Li et al., 2013; Mättö et al., 2005; Maukonen et al., 2006; Scanlan et al., 2006; Kong et al., 2012; Li L et al., 2016). Here, contrary to our hypothesis, the overall diversity within an individual, as well as variability in community structure and membership across time did not differ between children with ASD and unaffected controls. Likewise, the previously reported temporal changes in the α- and β-diversity of study populations were not observed in either group (Flores et al., 2014). Yet, other studies have reported that the microbial community might be relatively stable

over time (Huttenhower et al., 2012; Costello et al., 2009; Martínez et al., 2013a). Even though overall microbial structure did not change significantly, changes in individual bacteria as well as in microbial metabolites were observed. In children with ASD changes in the abundance of bacteria at the order (e.g., Streptophyta), family (e.g., Clostridiaceae) and genus (e.g., *Clostridium*, *Roseburia*) level were detected. The interest of Clostridaceae and *Clostridium* in ASD symptomology has previously been described (Chapter 2). Briefly, higher abundances of *Clostridium* are often observed in the ASD population (De Angelis et al., 2013; Grimaldi et al., 2017) and it has been hypothesized that *Clostridium* can contribute to ASD symptoms potentially due to the production of entereo- and neurotoxins (Finegold, 2008). Other bacteria that were shown to increase over the 6-month study period could also have health implications. Higher levels of *Dialister* were reported in children with appendicitis (Jackson et al., 2014) and it has been found to be one of the most abundance bacterial taxa in IBD (Lopetuso et al., 2008). Additionally, increased abundance of *Dialister* was associated with increased temperament in boys (Christian et al., 2015). On the other hand, *Roseburia*, which decreased in children with ASD, has been recognized for its ability to affect colonic motility, maintain immunity and exerting anti-inflammatory properties through the production of butyrate (Tamanai-Shacoori et al., 2017) and positive associations with mood have been reported in adults (Li L et al., 2016). The physiological significance of these bacterial changes observed in this ASD population, especially *Clostridium*, is unknown. Here, we did not see changes in the symptom severity of social deficits or GI symptoms. Thus, future studies potentially collecting samples more frequently and including a larger sample size are needed to investigate temporal variability in children with ASD and the impact on health outcomes.

Remarkably, we also see changes in all VFA concentrations in children with ASD, but only increases in acetate concentration in CONT children. These variations could be due to changes in the capacity of the bacteria to produce VFAs as well as to changes in absorption by the host. Here, we did not find significant changes in the mmDA and BCoAT genes in children with ASD, suggesting that the decrease in fecal VFA concentrations could be due increased absorption by the host rather than decreased production by the bacteria. Previous research has indicated that considering stability based on microbiota function is important (Lozupone et al., 2012), as compositional changes might not reflect functional changes due to functional redundancy (Moya & Ferrer, 2016). Changes in microbial metabolites such as VFAs could have important health implications since associations between host health and VFAs have been established (Macfarlane & Macfarlane, 2012). Higher levels of VFAs were observed in children with ASD (Wang et al., 2012) and it was demonstrated that isovalerate correlated with symptoms of depression (Szczesniak et al., 2015). The structural similarity between valerate and the neurotransmitter GABA (Lacher et al., 2007) makes valerate a potential candidate to interfere with neurotransmission (Szczesniak et al., 2015). Even though it remains to be elucidated whether isobutyrate is harmful or beneficial to colonocytes, it has been demonstrated that isobutyrate can be metabolized under low butyrate conditions (Jaskiewicz et al., 1996). Thus, fluctuations in VFA concentrations could be associated with host responses due to the impact of VFAs on GI health (Clausen et al., 1995) and the potential as serving as a messenger in the communication between the GI microbiota and brain (Berding & Donovan, 2016). However, no changes in social deficit or GI symptoms were not detected herein; thus, future studies are needed to elucidate the potential physiological importance of VFA fluctuations in children with ASD.

Some studies have aimed at identifying bacterial taxa that could potentially contribute to a more stable microbiome (Flores et al., 2014; Galloway-Peña et al., 2017). These studies have found that a higher temporal stability of oral and GI microbiome in cancer was associated with presence of commensal bacteria such as *Akkermansia* and absence of pathogenic-associated bacteria such as *Staphylococcus* and *Streptococcus* (Galloway-Peña et al., 2017). Here, we observe that children with ASD with a more stable microbiome harbored a distinct microbial community compared to children with a less stable microbiome. Thereby, destabilizing bacteria, such as Coriobacteriaceae, could be associated with pathogenic effects. Species within the Coriobacteriaceae family could potentially disrupt the epithelial barrier and lead to barrier dysfunction by colonizing the mucosal surfaces and affecting the epithelial cell metabolism (Clavel et al., 2014; Stenman et al., 2012; Turnbaugh, 2012). Furthermore, Coriobacteriaceae was positively correlated with reactive oxygen species and might be involved in the host inflammatory status and chronic inflammatory conditions (Qasem et al., 2017; Clavel et al., 2014; Chen et al., 2012; Zhang et al., 2009). On the other hand, microbial genera with a potentially stabilizing effect in the ASD population (i.e., *Lactococcus*, *Turicibacter*, *Phascolartobacterium*, *Dorea*) were described in the literature as beneficial symbionts. Lactic acid bacteria such as *Lactococcus* could have beneficial functions through production of anti-microbial compounds, modulation of the host's immune response (Soomro et al., 2002; Lukjancenko et al., 2012) and counteracting the virulence factor of pathogenic bacteria such as *Staphylococcus aureus* (Nouaille et al., 2014). Low abundance of *Phascolartobacterium* was associated with the presence of inflammation in IBD (Bajer et al., 2017) and *Dorea* was associated with better cognition and decreased inflammation in a model of hepatic

encephalopathy (Bajaj et al., 2012), suggesting potential anti-inflammatory effects of these genera.

Surprisingly, in this cohort Clostridiales was more abundant in children with ASD and a more stable microbiota. Negative health effects have been associated with Clostridiales and *Clostridium* species within this order and the abundances has have previously been linked to ASD (Luna et al., 2017). Additionally, abundance of Clostridiales was shown to be increased in children with ASD who developed GI symptoms at or around the same time of ASD diagnosis, suggesting that this order could potentially contribute to symptom development (Williams et al., 2011). Previous studies have reported that Clostridiaceae, a family within the order of Clostridiales, was more prevalent in adults with a more variable microbiota composition over a 3-month period (Flores et al., 2014). However, other genera and species within Clostridiales could potentially have beneficial effects. For example, it has been shown that the decrease in one member of the Clostridiales order (Clostridiales Incertae Sedis XI) could be associated with the development of *C. difficile* infections (Vincent et al., 2013). Clostridiales also includes sensitive oxygen species which could potentially be associated with improving GI barrier function and reducing the production of reactive oxygen species in inflammatory states (Darnaud et al., 2017; Ma et al., 2018). Additionally, species within Clostridiales can stimulate the production of cytokines and induce regulatory T-cells which can improve intestinal dysbiosis, reduce inflammation and protect against colitis and allergic responses (Narushima et al., 2014; Atarashi et al., 2013) Commensal genera within the order of Clostridiales include *Coprococcus*, *Blautia*, *Pseudobutyrivirbrio*, *Ruminococcus*, *Roseburia* and *Oscillospira* which have been associated with health benefits and can ferment carbohydrates and amino acids to produce anti-inflammatory metabolites (Stefka et al., 2014). Thus, future studies at the species level are

required to delineate which species within the Clostridiales order could potentially promote a more stable microbiome in children with ASD. Likewise, further research is warranted to elucidate whether a stable microbiota is more beneficial in children with ASD. Since Clostridiales has been associated with ASD symptomology, it could be hypothesized that a stable microbiota that is high in bacteria such as Clostridiales might actually be less beneficial in children with ASD.

In the unaffected control group, different bacterial taxa were identified as stabilizing and destabilizing bacteria. Pathogenic bacteria such as Enterobacteriaceae contributed more to an unstable microbiota whereas bacteria generally regarded as beneficial such as Barnesiellaceae, *Adlercreutzia* and *Facelibacterium* contributed more to a stable microbiota. A microbiota rich in Barnesiellaceae might be protective against bloodstream infections (Montassier et al., 2016) and *Adlercreutzia* abundance was associated with leanness (Ziętak et al., 2016). Likewise, *Faecalibacterium* is usually regarded as a beneficial bacterium due to its anti-inflammatory properties (Quevrain et al., 2015), improvements of GI barrier function (Carlsson et al., 2013) and support of mucosal immune homeostasis (Hornef & Pabst, 2016). Similar to previously published literature, species diversity and richness was associated with a more stable microbiota over the 6-month period in unaffected control children (Flores et al., 2014). Surprisingly, this relationship was not observed in children with ASD. Thus, further investigation of this association is warranted, but it could indicate that diversity might affect stability differently depending on the health state of the host.

Besides only investigating changes in the microbiota, we were also interested in analyzing whether microbial stability in children with ASD can be associated with symptom severity as previous studies have demonstrated that microbial stability could be associated with

health outcomes such as functional bowel disorders (Maukonen et al., 2006; Mättö et al., 2005). Contrary to our hypothesis we observed that children with ASD who have less changes in presence and absence of OTUs (based on unweighted UniFrac distance) had higher social deficit scores. Although less variability is considered to be more beneficial due to the ability to maintain bacterial functions over time and to resist pathogen invasion (Coyte et al., 2015), less variability in ASD could correspond to a constant presence of pathogenic bacteria that could affect symptomology. Appearance and disappearance of some bacteria has previously been reported (Martínez et al., 2013) and here, although not statistically different, we observe that some bacterial genera such as *Sarcina*, *Leuconostoc* or *Megamonas* were not detected at some time points but were detected at other time points in children with ASD.

Investigating variations in bacterial taxa associated with symptom severity could potentially provide evidence for interventions to promote health by targeting the stability of specific bacteria. Here, we provide preliminary evidence for an association between changes in bacterial taxa and ASD symptoms. First, when comparing children with ASD based on social deficit symptom severity, different patterns of changes of bacteria in children with moderate vs. severe symptoms were observed. For example, *Clostridium* abundance decreased over the 6-month period in children with moderate symptoms, but didn't show significant variations in children with severe symptoms. Second, different health implications could be attributed to the bacteria that changed in children with moderate or severe social deficits. For example, *Sarcina* which decreased in the moderate severity group was associated with infection in Celiac Disease (Karakuş & Lırsaçlıoğlu, 2014) and could contribute to disease in humans (Lam-Himlin et al., 2011). *Methanobrevibacter* which increased in children with severe symptoms has been linked to constipation (Lurie-Weinberger & Gophna, 2015), flatulence (Seo et al., 2017) and could

contribute to development of chronic constipation in IBS (Pimentel et al., 2012). It is also interesting to note that no changes in nutritional intake were observed in children with severe ASD symptoms. Although this could be due to the relatively small group size (n=7), it could also indicate that changes in the microbiota in severe cases of ASD could be independent of dietary intake and might be inherent to ASD itself. Once again, future studies with a bigger sample size are required to further explore this relationship.

Next, we set to explore the impact of diet on the microbial stability in children with ASD in order to identify potential environmental influences in promoting microbial stability. Therefore, we employed several analyzes on the microbial stability in the context of diet, dietary patterns and introduction of specialty diet to shed some light on the diet-microbiota stability relationship. Unique microbial profiles based on dietary patterns and specialty diets were observed and food groups associated with a more stable microbiota composition were identified.

First, we provide preliminary evidence that dietary interventions were associated with some temporal microbial variability in this cohort of children with ASD. The interest in studying the effect of specialty diets on the GI microbiota stems for the wide usage of these diets among individuals with ASD. Due to the lack of effective medical treatment approaches, parents often seek alternative treatment options for their children after ASD diagnosis. Although there is often no convincing justification for the efficacy of alternative treatments, dietary changes that are considered risk free by the lay public are often adopted (Stewart et al., 2015). It has been estimated that one-third of children have been treated with some dietary intervention after of ASD diagnosis (Levy et al., 2003). A number of nutrition intervention strategies including gluten-free/casein-free (GF/CF) diet, ketogenic diet, or Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols (FODMAP) diet have been explored to treat

behavioral symptoms and comorbid GI distress. Some dietary interventions specifically focus on targeting the GI microbiota in order to manage some symptoms of ASD. For example, the purpose of the specific carbohydrate diet (excludes grains, processed foods, starchy vegetables, canned vegetables, flour, sweeteners and milk products), which is used by some parents to manage symptoms of ASD, is to alleviate symptoms of malabsorption and prevent growth of potentially pathogenic bacteria which could potentially affect symptomology (Gotschall, 2004). Delineating how these diets shape on the GI microbiota could provide information on the potential mechanisms whereby dietary interventions affect some symptoms of ASD. In healthy individuals, following a gluten-free diet caused a decrease in beneficial bacteria such as *Bifidobacterium*, *C. lotuseburense*, *F. prausnitzii* and *Lactobacillus* and an increase in pathogenic bacteria including Enterobacteriaceae and *E. coli* (De Palma et al., 2010; Singh et al., 2017). Likewise, the ketogenic diet can reduce total microbiota abundance and composition in a mice model (Newell et al., 2016) and following a low FODMAP diet could reduce the abundance of total bacteria as well as *Akkermansia muciniphila*, *Bifidobacterium* or Actinobacteria (Halmos et al., 2014; Bennet et al., 2017). Previous studies investigating the microbiota composition in children with ASD have collected information in dietary interventions of the subjects, but did not investigate its potential impact on the GI microbiota composition (Horvath et al., 1999; Finegold et al., 2002; Parracho et al., 2005; Wang et al., 2011; Kang et al., 2013) Here, four children with ASD were following a specialty diet at the 6-week sample collection and seven children at 6-month time point, with the most prominent specialty diet being the GF/CF diet. Even though microbial structure and membership were not influenced by a specialty diet status in this cohort, the introduction of a dietary intervention could have induced some potentially favorable changes in children with ASD such as the increase in Actinobacteria

and *Bifidobacterium*. *Bifidobacterium* is generally regarded as beneficial (Patten & Laws, 2015) and Actinobacteria was related to protection against pathogens and maintenance of the immune system (Gosalbes et al., 2011). Regarding VFA concentrations, reduction of acetate concentrations seemed to have been driven more significantly by introduction of specialty diets whereas the concentrations of propionate, butyrate and isobutyrate decreased independent of introduction of specialty diet. Since these microbial metabolites could be involved in the microbiota-to-brain communication in ASD, future research is needed to determine whether dietary interventions can decrease these communicators on a physiologically relevant level. Because of the low sample number and differences in dietary interventions among the children, it is difficult to define whether the microbiota might be a moderator of dietary interventions used to manage ASD symptoms. Additionally, even though changes in the microbiota composition based on specialty diet were demonstrated herein, no changes in symptoms of ASD were observed. Thus, more research is needed to determine whether these diets can be used as a way to manage some symptoms of ASD through the microbiota composition.

Second, since previous studies have shown that a dietary pattern characterized by intake of healthier foods was associated with greater microbial stability in healthy children (Berding et al., 2018), we investigated in more detail which dietary factors could be linked to microbial variability in children with ASD. In children below the median in the healthier DP1, reduction of species richness and diversity was observed, suggesting that the diversity within an individual could be more impacted in children scoring low on a healthy eating pattern. Moreover, community structure measured by weighted UniFrac distance tended to change more in children above the median in DP2-ASD, indicating that an unhealthier dietary pattern rich in animal-based and processed foods might have a more significant impact on the relative abundance of

bacteria in this population. This observation is in accordance with previous literature suggesting that the relative abundance of bacterial taxa that are persistently present in the GI tract might be a more significant contributor to temporal dynamics (Flores et al., 2014). Furthermore, animal-based diets were shown to increase β-diversity in humans (David et al., 2014) and individuals following a Western-diet had a decreased diversity compared to individuals following a plant-based diet (Conlon & Bird, 2014).

Although only a few bacterial taxa were changed in association with dietary patterns in children with ASD, some of the shifts were distinct for each dietary pattern. For example, a decreased intake of healthy food such as fruit and vegetables in children above the median in DP1-ASD coincided with an increase in potentially pathogenic bacteria such as Erysipelotrichaceae. Erysipelotrichaceae could be correlated with inflammation (Kaakoush, 2015) and immunomodulation (Palm et al., 2014) and was more abundant in patients with intestinal diseases (Mancabelli et al., 2017). Interstingly, the abundance of Erysipelotrichaceae decreased in children above the median in DP2. Likewise, DP2-ASD was associated with changes in Cyanobacteria. Little is known about the function of Cyanobacteria in the human GI tract, but some evidence suggests that species within this phylum might be able to produce neurotoxins that might contribute to neurological disease in humans (Brenner, 2013). Furthermore, differences in VFA concentrations were observed based on dietary patterns, suggesting that the overall the functional capability to produce VFA could also be affected by habitual dietary intake in children with ASD.

Although unique microbial associations were observed based on two dietary patterns in children with ASD, the decrease in abundances of *Oscillospira* and *Clostridium* were associated with both dietary patterns. The observation that the abundance of *Clostridium* varied over the 6-

month study period seemingly independent of dietary factors could suggest that *Clostridium* abundance potentially the impact on ASD symptoms might depend on other factors besides diet. Future investigations into this relationship are required to determine which factors could potentially influence the temporal variability of *Clostridium*. Likewise, no associations with social deficits scores were observed, so that it is not possible at this point to draw conclusions regarding the physiological importance of temporal *Clostridium* variations in the ASD population.

Even though two similar dietary patterns were observed in CONT children, namely a less dietary pattern including food groups such as sweets, fried food, snacks and sweetened beverages, and one healthier dietary pattern that included fish, vegetables, and fruit, different microbial profiles associated with these patterns were observed. The unhealthier dietary pattern in CONT children was associated with a decrease in *Proteobacteria* and *Slackia*, whereas the healthier dietary pattern was associated with a decrease in *Parabacteroides*, *Butyrivirbio* and *Veillonella*. Additionally, when analyzing the association between diet and microbiota structure based on weighted UniFrac distance, dietary components that to contribute to a more or less stable microbial community differed in the groups. Whereas whole grains, vegetables, fish and condiments contributed to a stable microbiota in control children, in children with ASD fish and total protein intake contributed more to a stable microbiota, but snacks and condiments were more significantly associated with an unstable microbiota. Thus, if different dietary components can contribute differently to the microbiota stability based on different health statuses warrants further investigation.

Overall, less variations than expected were observed in overall structure and abundance of bacterial taxa in children with ASD. The lack of significant changes in individual bacterial

taxa could be explained by the relative stability of the microbiota based on α- and β- diversity matrices. Additionally, the lack of significant variations could be due to the small sample size as well as the limited amount of samples collected for each participant and the time between collection time points. Additionally, since no associations with GI symptoms or social deficit scores were observed throughout the analyses, the physiological relevance of the temporal variability observed in microbial taxa and metabolites remains to be determined. The lack of association could be due to the relative stability of ASD symptoms over time. A recent meta-analysis suggests that ASD symptoms might be remarkably stable over time (Bieleninik et al., 2017); thus, as expected overall ASD symptoms did not change during the study period. Likewise, the lack of associations with social deficit scores could be due to the potential stronger impact of the microbiota on other symptoms of ASD (i.e., repetitive behaviors) as well as on other associated symptoms (e.g., irritability). Future studies including a larger number of subjects with more frequent stool sampling and measuring other symptoms of ASD are needed to further identify the microbial stability in this population and to analyze how microbial variability potentially impacts symptoms of ASD. Likewise, some changes in microbiota composition observed in the ASD population as a whole did not change based on dietary patterns or specialty diet. Thus, future studies should explore other factors that could potentially contribute to these changes.

Despite these limitations, the results reported herein provide preliminary evidence that the microbiota composition of children with ASD can vary over time, that diet can play an important role in determining stability of microbiota in this population and that varaibiaity might be associated with some symptoms associated with ASD. Future studies investigating the longitudinal dynamics in the GI microbiota and factors contributing to stability or instability are

warranted. For example, in this study we observe that the variability in abundance of some bacteria was only associated with diet or symptom severity, but the abundance of other bacteria (e.g., *Clostridium*) was significantly associated with all categories investigated herein. Our analyses are strengthened by including factors known to influence thee microbiota composition, such as changes in dietary intake and medication (Maier & Typass, 2017) as covariates in the longitudinal analysis. To the best of our knowledge, this is the first study investigating longitudinal dynamics of the GI microbiota in children with ASD. Understanding the environmental factors determining the functional and compositional changes in the GI microbiota could provide important evidence for the development of intervention strategies for microbiota-associated diseases such as ASD.

6.5 Tables and Figures

Table 6.1 Enrollment and attrition rate of study

Group	Number of subjects enrolled	Number of Samples collected at Baseline	Number of samples collected at 6 weeks	Number of samples collected at 6 months
Control	34	32	32	29
ASD	41	26	25	22

Data expressed as n

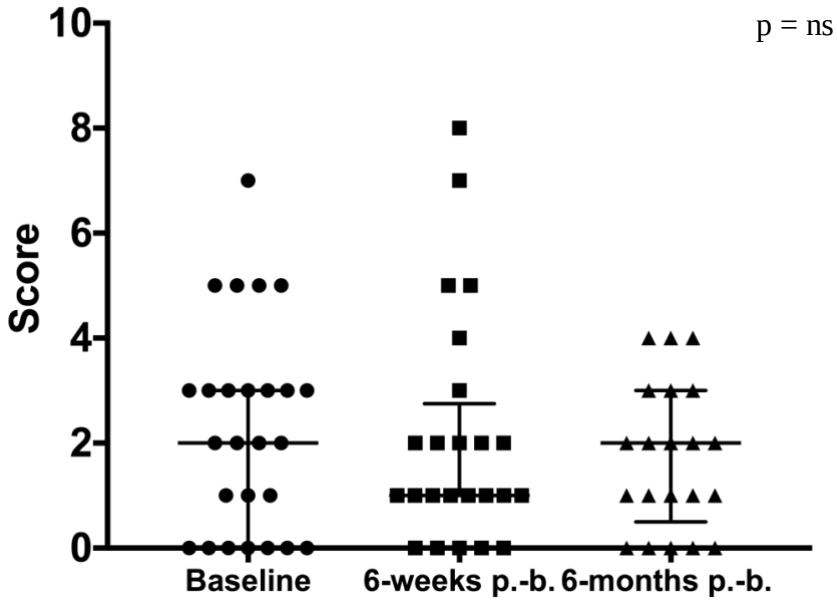
Table 6.2 Percentage of participants introducing medication, specialty diets or probiotics over the study period

	Medication use in month before sample collection		**Following Specialty Diet at sample collection**		**Probiotic use in month before sample collection**	
Group	6 Weeks	6 Months	6 Weeks	6 Months	6 Weeks	6 Months
ASD	1	3	4	7	0	0
CONT	3	1	0	0	0	0

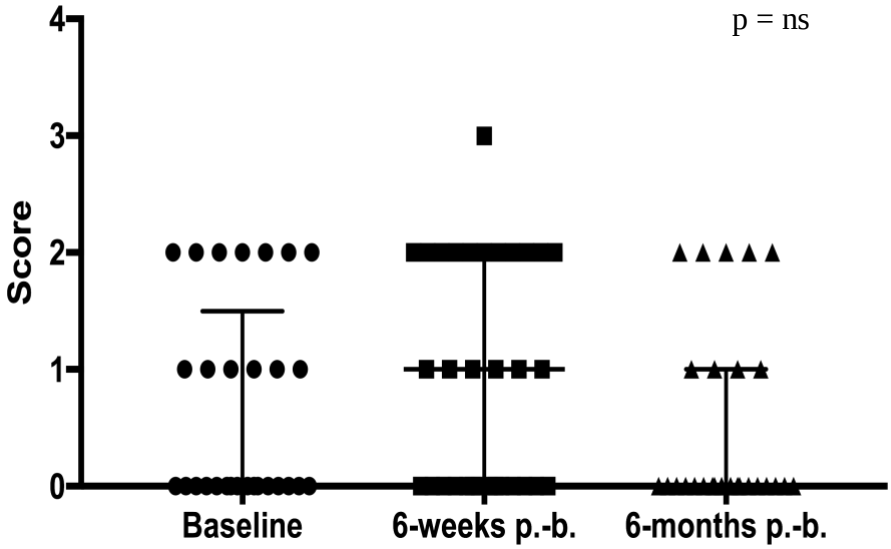
Data shown as n

Figure 6.1 GI severity index scores[1] over 6-month period in children with ASD and controls

A) ASD



B) CONT



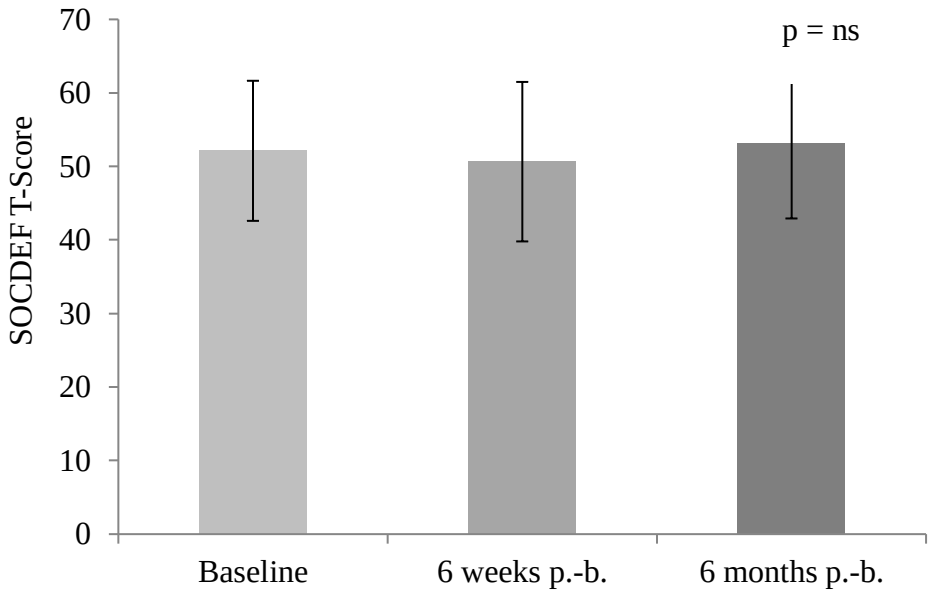
Data expressed as median (IQR)
[1]GI severity scores were derived from the GI severity index (possible range 0-7);
CONT, unaffected controls; ASD, Autism Spectrum Disorder; p.-b. post-baseline

Table 6.3 Stool consistency in children with ASD (ASD) and unaffected controls (CONT) over a 6-month period

	ASD			CONT		
Stool Consistency[1]	**Baseline (n=26)**	**6-weeks p.-b. (n=25)**	**6 months p.-b. (n=22)**	**Baseline (n=32)**	**6-weeks p.-b. (n=32)**	**6 months p.-b. (n=29)**
Type 1 (separate hard lumps)	0	0	1	1	1	1
Type 2 (sausage shaped but lumpy)	5	3	2	3	8	3
Type 3 (sausage-shaped with cracks on surface)	5	6	4	13	9	12
Type 4 (smooth and soft)	12	13	12	15	14	11
Type 5 (soft blobs)	2	0	2	0	0	0
Type 6 (mushy)	2	1	0	0	0	1
Type 7 (watery)	0	0	0	0	0	0

[1]Stool consistency was measured using the Bristol Stool chart; Data expressed as n; no statistical difference in stool consistency over 6 month period in either group

Figure 6.2 SOCDEF[1] T-scores scores over 6-month period in children with ASD



Data expressed as mean ± SD
[1]Social deficit score measured by PDDBI-SV

Table 6.4 Nutrient and food group intake at baseline, 6-weeks post baseline and 6-months post-baseline for children with ASD (ASD) and controls (CONT)

a) Nutrient intake that differed significantly across time in children with ASD (ASD)

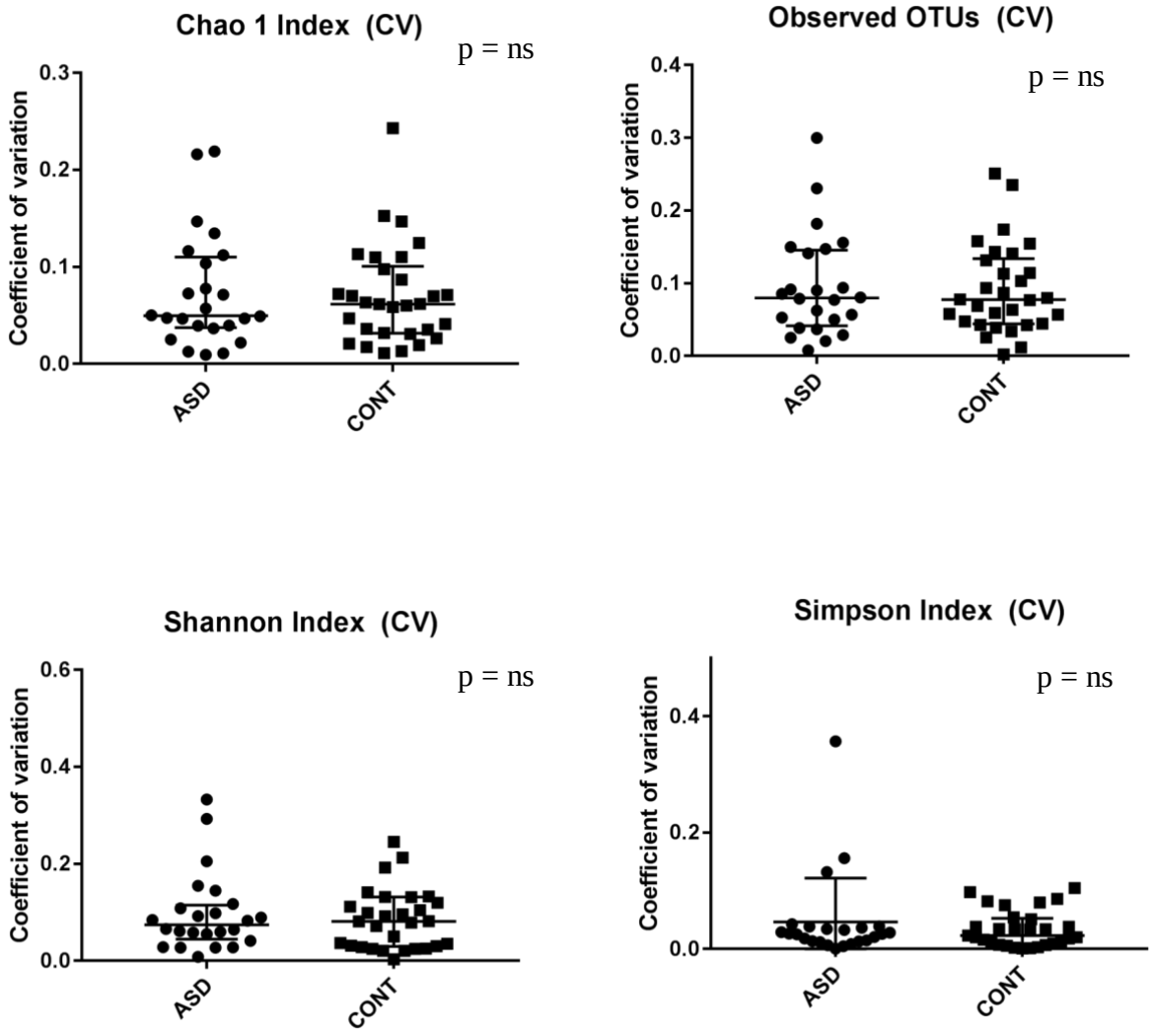
Nutrient Intake	**Baseline (n=26)**	**6-weeks p.-b. (n=25)**	**6 months p.-b. (n=22)**
Macronutrients			
Total carbohydrate (g)	175 (147-220)	171 (135 (221)	149 (109-201)*
Total sugars (g)	81 (56-94)	71 (58-92)	61 (41-90)*
Added sugars (g)	36 (23-42)	33 (23-52)	19 (12-27)*
Micronutrients			
Vitamin A (μg)	435 (228-651)	337 (221-485)	259 (160-328)*
Vitamin E (mg)	6.2 (4.2-8.1)	5.7 (3.8-7.0)	5.1 (3.5-6.3)*
Magnesium (mg)	169 (132-231)	148 (124-226)	143 (127-194)†

b) Food and Nutrient intake that differed significantly across time CONT

Food Group (servings per day)	**Baseline (n=32)**	**6-weeks p.-b. (n=32)**	**6 months p.-b. (n=29)**
Starchy Vegetables	0.4 (0.3-0.6)	0.5 (0.4-1.8)	0.6 (0.5-0.8)*
Protein Food	1.0 (0.7-1.6)	1.6 (1.1-2.3)	1.3 (1.1-1.9)*

Data expressed as median (IQR); p-value in same group and row indicated differences at *p≤0.05; †p≤0.1; Food groups derived from Youth and Adolescence Food Frequency questionnaire; nutrient intake derived from 3-day food diary; p.b. post baseline

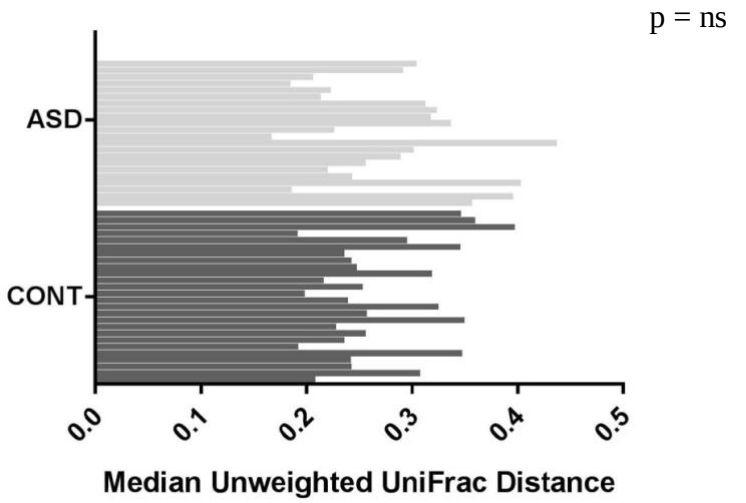
Figure 6.3 Temporal variability as measured by coefficient of variation (CV) in diversity within each individual in children with ASD (ASD) and unaffected controls (CONT)



Data expressed as median (IQR)

Figure 6.4 Plots of variability measured by unweighted (A) and weighted UniFrac (B) distance within each individual in children with ASD (ASD) and unaffected controls (CONT)

A)



B)

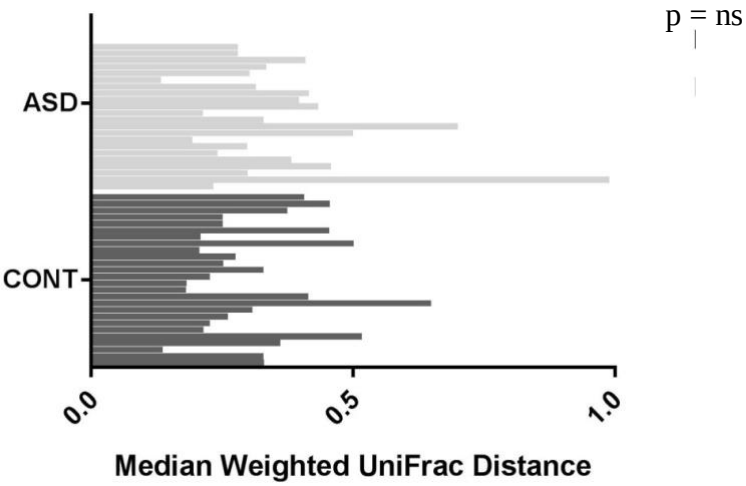


Table 6.5 Measures of relative abundance of bacterial genera in feces that differed significantly between baseline, 6 weeks post-baseline and 6 months post-baseline in children with ASD (ASD) and unaffected controls (CONT)

a) Bacterial variations in children with ASD

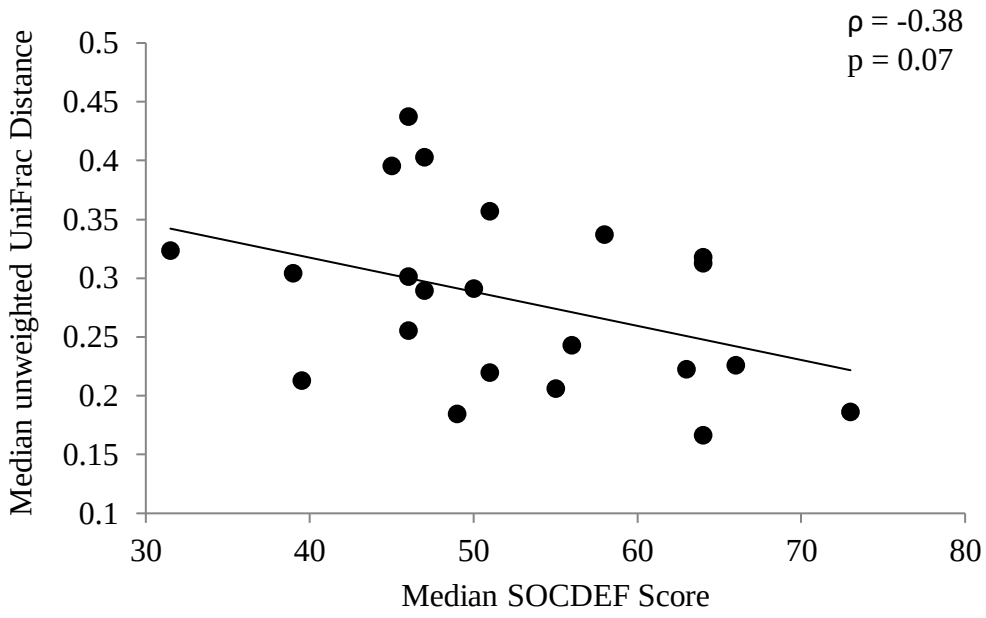
Bacterial taxa (% of sequences)	**Baseline (n=26)**	**6-weeks p.-b. (n=25)**	**6 months p.-b. (n=22)**
Bacterial order			
Streptophyta	0 (0-0.001)	0 (0-0.005)	0.002 (0-0.02)†
Bacterial family			
Clostridiaceae	0.28 (0.14-1.5)	0.53 (0.16-1.47)	0.25 (0.08-0.49)*
Bacterial genus			
Firmicutes			
Clostridaceae_ Clostridium	0.33 (0.05-0.66)	0.19 (0.07-0.42)	0.06 (0.03-0.14)*
Roseburia	0.46 (0.27-0.72)	0.58 (0.36-0.89)	0.29 (0.14-0.89)*
Dialister	0.12 (0.007-0.72)	0.13 (0.007-1.5)	0.02 (0.005-1.9)†

b) Bacterial variations in CONT

Bacterial order (% of sequences)	**Baseline (n=32)**	**6-weeks p.-b. (n=32)**	**6 months p.-b. (n=29)**
Bacterial family			
Enterobacteriaceae	0.02 (0.007-0.12)	0.02 (0.01-0.39)	0.03 (0.005-0.11)†
Bacterial genus			
Firmicutes			
Enterococcus	0 (0-0.001)	0 (0-0.002)	0.001 (0-0.002)†

Data expressed as median (IQR); within same group and row, significant at *$p \leq 0.05$; †$p \leq 0.1$

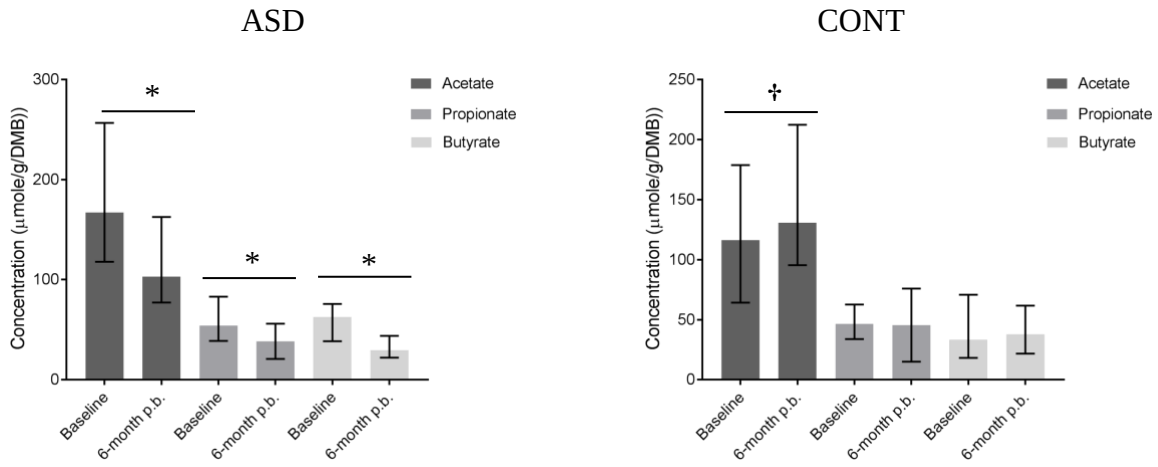
Figure 6.5 Association between social deficit scores and temporal variability based on unweighted UniFrac distance



Correlation was assessed using Spearman Rank correlation

Figure 6.6 VFA concentrations at baseline and 6-months p.-b. in children with ASD (ASD) and unaffected controls (CONT)

a) SCFA



b) BCFA

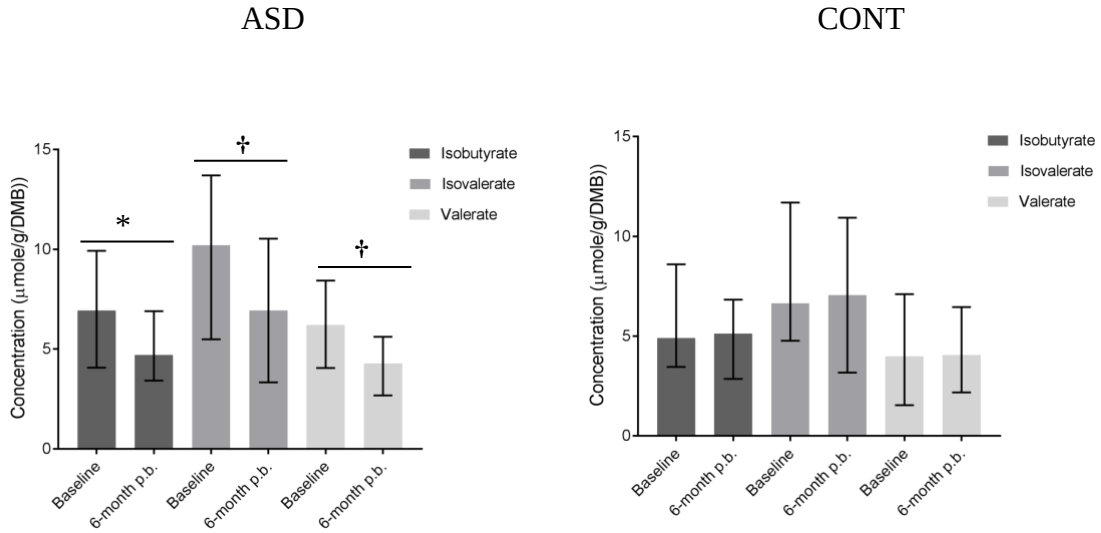
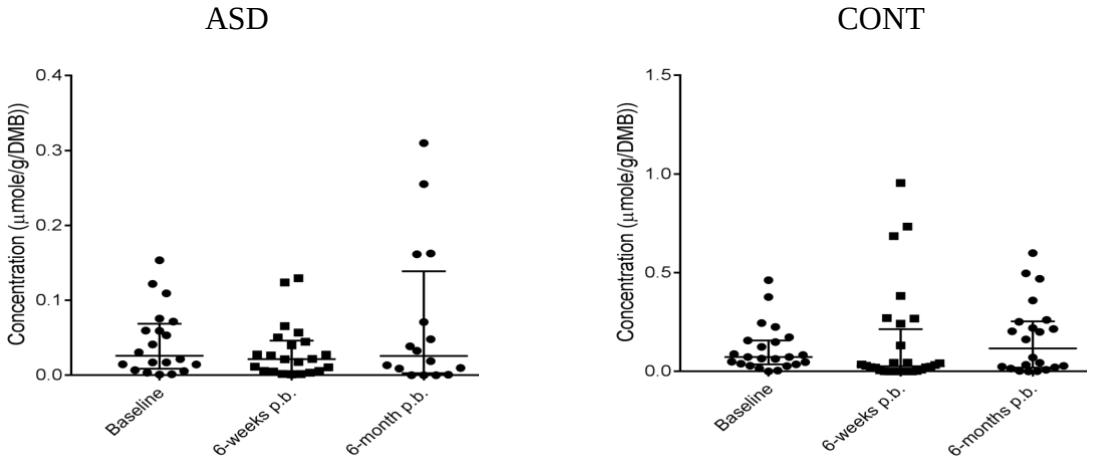
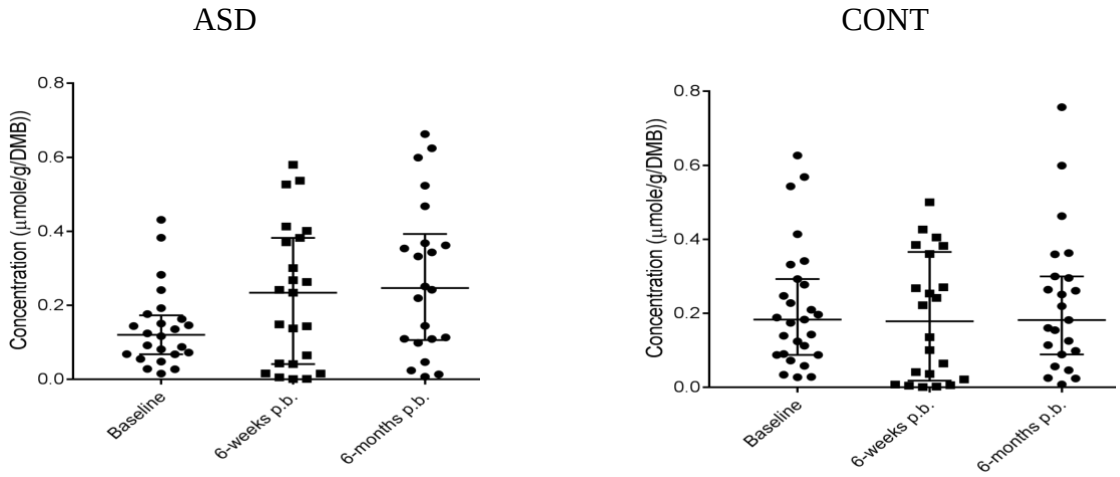


Figure 6.6 (cont.)

C) mmDA



D) BCoAT



Median (IQR); *p≤0.05; †p≤0.1
CONT, unaffected controls; ASD, Autism Spectrum Disorder; DMB, dry matter basis; SCFA, short chain fatty acids; BCFA, branch chain fatty acids; mmDa, methylmalonyl CoA decarboxylase; BCoAT, butyryl-CoA:acetate acyltransferase; p.-b., post-baseline

Figure 6.7 Differences in bacterial taxa among stability categories in children with ASD

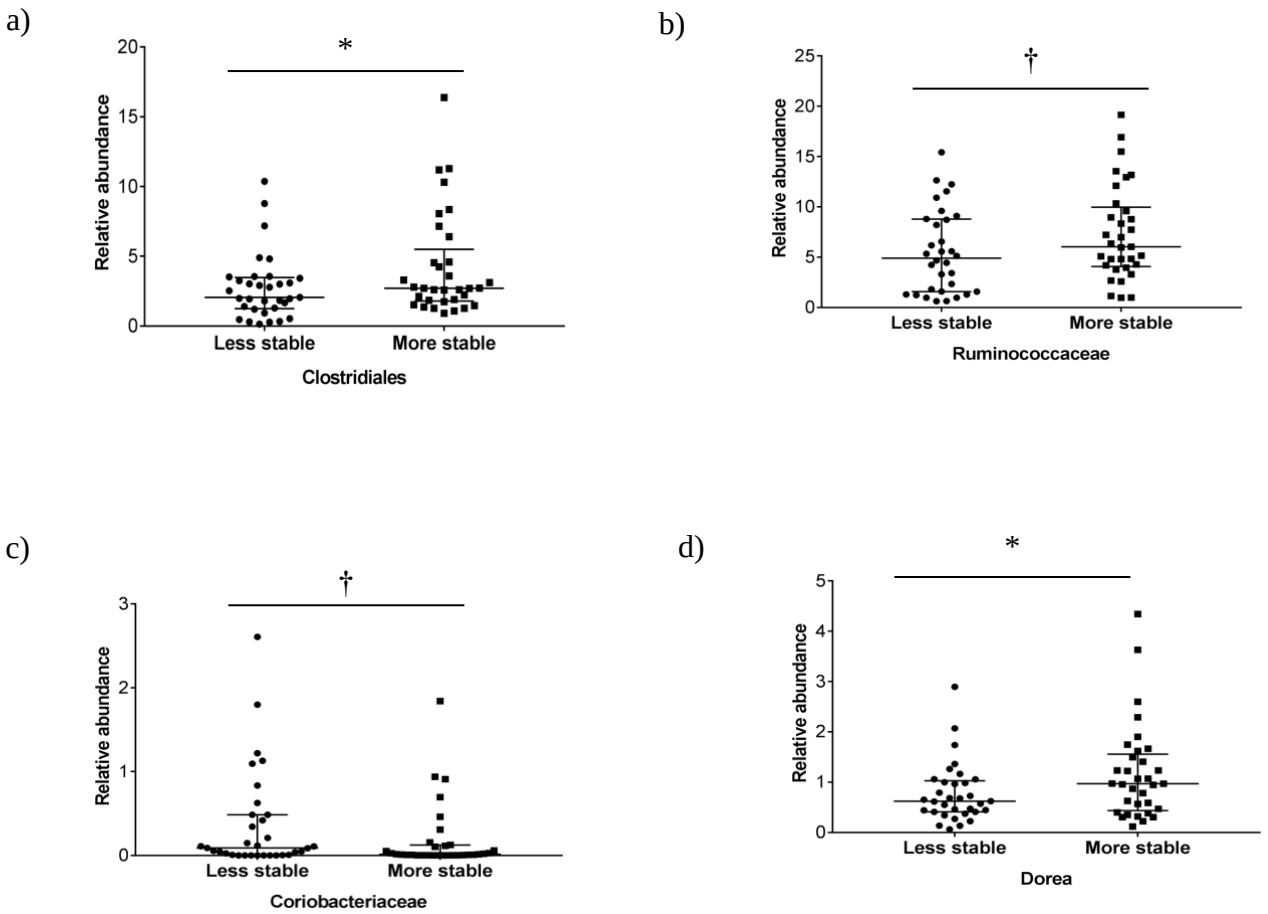
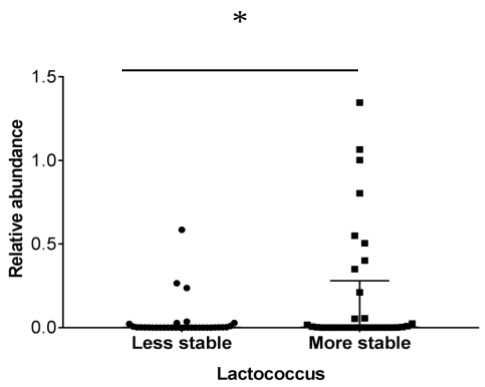
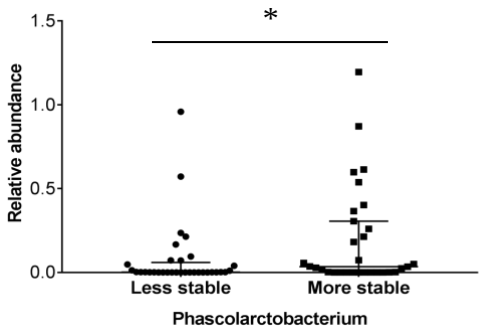


Figure 6.7 (cont.)

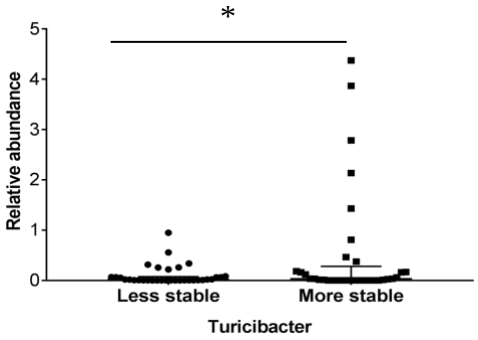
e)



f)



g)



Data expressed as median (IQR); * p≤0.05; †p≤0.1; Individuals in the ASD group were assigned to a category of stability based on falling above or below the median of median weighted UniFrac distances

Table 6.6 Bacterial abundance and nutrient and food groups contributing to stability categories in unaffected controls

	Less stable (n=13)	More stable (n=13)
Bacterial richness/diversity		
Chao 1 Index	446 (410-482)	473 (413-507)†
Shannon Index	4.9 (4.3-5.5)	5.2 (4.8-5.6)†
Bacterial abundance (% of sequences)		
Verrucomicrobia	1.88 (0.03-10.8)	0.4 (0.01-1.3)*
Adlercreutzia	0.004 (0-0.07)	0.06 (0.001-0.19)*
Faecalibacterium	8.7 (5.9-12.8)	12.9 (8.5-18.4)*
Sutterella	0.26 (0.003-0.63)	0.31 (0.13-1.65)†
Bilophila	0 (0-0.1)	0.1 (0-0.07)†
Akkermansia	1.88 (0.03-10.7)	0.39 (0.01-1.3)*
Food group (servings/day)[1]		
Vegetables	2.0 (1.8-3.2)	2.5 (1.8-3.1)†
Sweetened beverages	0.12 (0.08-1.7)	0.18 (0.08-0.26)†
Kid's meals	1.6 (1.1-2,1)	0.9 (0.5-1.3)*
Fish	0.06 (0.06-0.3)	0.18 (0.12-0.3)*
Condiments	0.16 (0.08-0.3)	0 (0-0.3)*
Nutrient[2]		
Whole grains (g)	0.84 (0.35-1.45)	0.95 (0.39-2.5)†

Data expressed as median (IQR); *p≤0.05; †p≤0.1; Individuals in the ASD group were assigned to a category of stability based on falling above or below the median of median weighted UniFrac distances

[1]Food groups derived from food frequency questionnaire

[2]Nutrient intake derived from 3-day food diary

Table 6.7 Nutrient and food groups contributing to stability categories in children with ASD

	Less stable (n=13)	**More stable (n=13)**
Food group (servings/day)[1]		
Snacks	1.0 (0.5-2.0)	0.7 (0.4-1.3)†
Fish	0 (0-0.14)	0.04 (0-0.14)*
Condiments	0.36 (0-0.5)	0.08 (0-0.4)*
Nutrient[2]		
Total protein (g)	43 (34-49)	52 (31-68)*

Data expressed as Median (IQR); *p≤0.05; †p≤0.1; Individuals in the ASD group were assigned to a category of stability based on falling above or below the median of median weighted UniFrac distances

[1]Food groups derived from food frequency questionnaire

[2]Nutrient intake derived from 3-day food diary

Table 6.8 Nutrient and food group intake in children with ASD at baseline, 6-weeks and 6-months post-baseline based on introduction of specialty diets

a) Changes in nutrients in children with ASD starting a specialty diet (n=7) during the study

	Baseline	**6-weeks p.-b.**	**6 months p.-b.**
Food group (servings/day)			
Vegetables	0.7 (0.3-1.6)	0.5 (0.3-0.9)	0.5 (0.2-0.9)†
Fish	0.06 (0.06-0.12)	0.12 (0.06-0.18)	0.18 (0.06-0.3)†
Nutrient intake			
Added sugars (g)	36 (23-44)	30 (23-41)	18 (13-24)*

b) Changes in nutrients in children with ASD without specialty diet (n=19)

	Baseline	**6-weeks p.-b.**	**6 months p.-b.**
Nutrient intake			
Vitamin E (μg)	3.8 (4.2-8.1)	6.0 (5.2-7.4)	5.2 (3.5-6.3)*
Folate (μg)	242 (201-311)	237 (160-315)	163 (134-235)†
Sodium (mg)	2010 (1687-2261)	1606 (1475-1957)	1887 (1468-2151)*

Data expressed as median (IQR); *p≤0.05; †p≤0.1 in same group and row significant; p.-b. post-baseline

Table 6.9 Microbiota composition and VFA concentration in children with ASD at baseline, 6-weeks and 6-months post baseline based on introduction of specialty diets

a) Changes in microbiota composition and VFA concentration in children with ASD following specialty diet (n=7)

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacteria (relative abundance)			
Actinobacteria	2.4 (0.6-6.3)	5.3 (1.4-8.3)	8.2 (4.1-9.5)*
Clostridiaceae	3.7 (0.09-10.2)	2.4 (0.6-5.7)	0.3 (0.06-1.8) †
Bifidobacterium	0.7 (0.5-1.3)	2.9 (1.1-5.8)	3.2 (0.03-1.8)*
VFA (µmole/g DMB)			
Acetate	142 (90-260)	.	84 (68-143)*

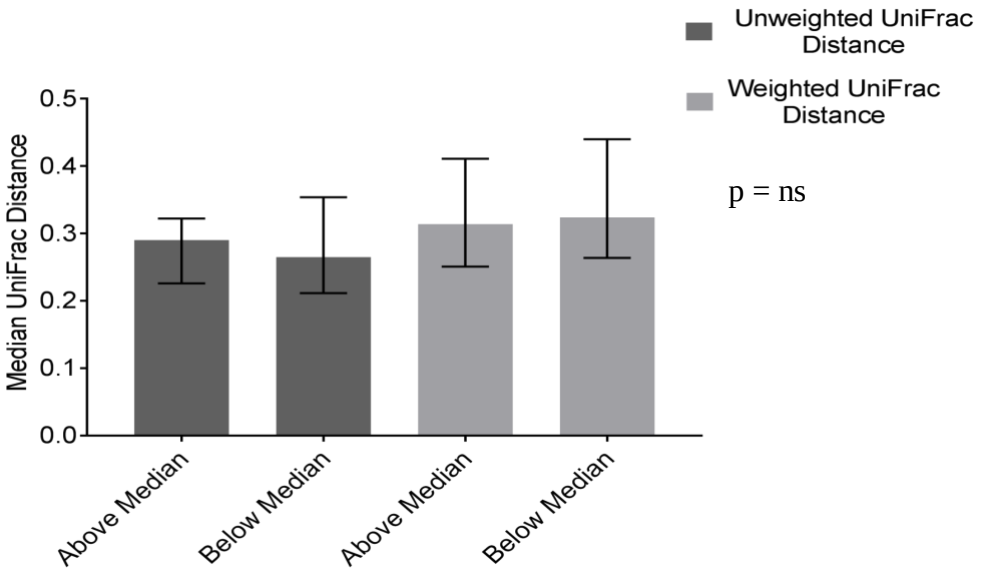
b) Changes in microbiota composition and VFA concentration in children with ASD not following specialty diet (n=19)

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacteria (relative abundance)			
Bacteroidetes	48.4 (17.5-55.6)	37.5 (15.0-46.9)	39 (1.1-44.7)*
Clostridium	0.2 (0.05-0.68)	0.1 (0.04-0.32)	0.05 (0.009-0.14)*
VFA (µmole/g DMB)			
Propionate	61 (50-84)	.	32 (28-59)*
Butyrate	64 (42-76)	.	59 (15-74)*
Isobutyrate	7.2 (3.6-10.0)	.	5.5 (4.2-9.9)*

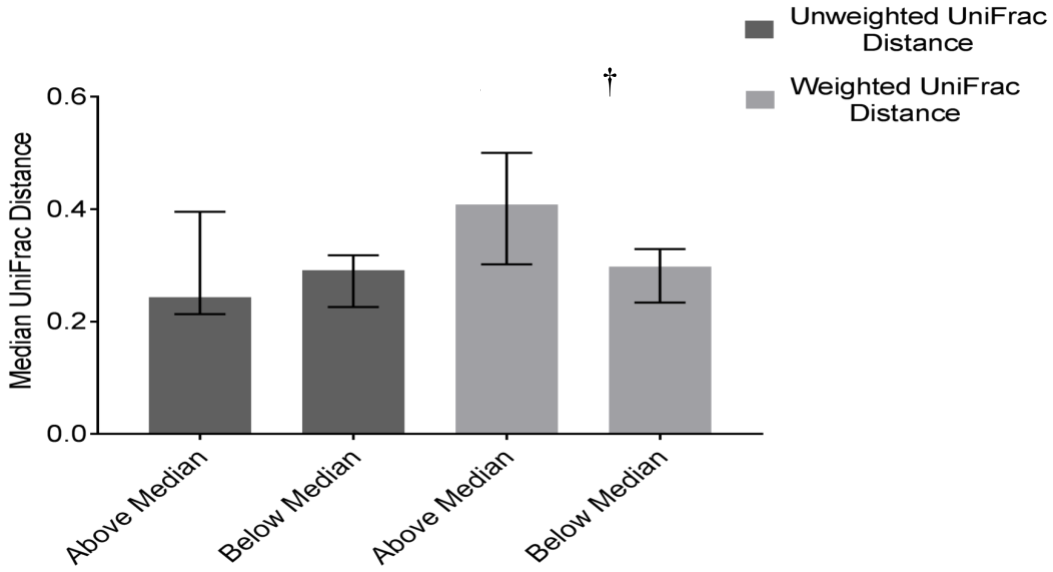
Data expressed as median (IQR); within same group and row, significant at *p≤0.05; †p≤0.1; VFA, volatile fatty acids

Figure 6.8 Differences in weighted and unweighted UniFrac distances based on dietary patterns in children with ASD

a) Dietary pattern 1



b) Dietary pattern 2



Median (IQR); †p≤0.1; dietary patterns were derived from a food frequency questionnaire using principal component and factor analysis (Chapter 5)

Table 6.10 Variation of coefficient of variation (CV) based on dietary patterns in children with ASD

	Dietary Pattern 1		Dietary Pattern 2	
Measure	**Above median (n=13)**	**Below median (n=13)**	**Above median (n=13)**	**Below median (n=13)**
Chao1 Index CV	0.05 (00.04-0.08)	0.05 (0.03-0.13)	0.05 (0.02-0.09)	0.05 (0.04-0.11)
Observed OTUs CV	0.08 (0.06-0.14)	0.08 (0.02-0.18)	0.07 (0.03-0.12)	0.08 (0.05-0.14)
Shannon Index CV	0.06 (0.05-0.09)	0.08 (0.04-0.14)	0.07 (0.06-0.1)	0.07 (0.03-0.13)
Simpson Index CV	0.03 (0.01-0.04)	0.03 (0.01-0.04)	0.02 (0.01-0.03)	0.03 0.01-0.04)

Data expressed as Median (IQR)

Dietary patterns were derived from the Youth and Adolescence Food Frequency Questionnaire using principal component and factor analysis (Chapter 5)

Table 6.11 Food group and nutrient intake at baseline, 6 weeks post-baseline and 6 months post-baseline based on dietary pattern in children with ASD

a) Dietary pattern 1 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food Group (servings per day)[1]			
Fruit	3.0 (2.7-3.6)	2.5 (1.6-2.7)	1.9 (1.4-2.7)*
Vegetables	1.7 (0.9-2.4)	0.7 (0.6-1.1)	0.9 (0.7-1.3)†
Juice	0.8 (0.0-0.9)	0.8 (0.06-0.8)	0.12 (0.06-0.51)†
Nutrient[2]			
Total carbohydrate (g)	194 (143-220)	173 (162-194)	133 (102-182)†
Added sugars (g)	31 (24-42)	36 (19-42)	21 (12-26)†

b) Dietary pattern 1 below median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food Group (servings per day)[1]			
Legumes, nuts and seeds	0.1 (0.1-0.16)	0.1 (0.1-0.3)	0.22 (0.1-0.9)*
Juice	0.84 (0.12-1.04)	0.8 (0.12-1.0)	1.0 (0.8-1.0)†
Refined carbohydrates	0.9 (0.5-1.1)	1.4 (1.2-1.5)	1.1 (0.9-1.9)*
Nutrient[2]			
Vitamin A (μg)	326 (277-428)	299 (170-473)	175 (81-445)*
Folate (μg)	242 (202-294)	182 (105-389)	195 (134-235)†
Phosphorus (mg)	792 (676-992)	616 (456-971)	646 (471-862)†
Refined grains (g)	4.6 (3.7-5.1)	3.3 (2.2-3.9)	3.5 (2.3-4.7†

Data expressed as median (IQR); *p≤0.05; †p≤0.1; p.b. – post-baseline; [1]Food groups derived from food frequency questionnaire; [2]Nutrient intake derived from 3-day food diary

Table 6.11 (cont.)

c) Dietary Pattern 2 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food group (servings per day)[1]			
Protein foods	1.8 (1.1-2.2)	1.6 (0.8-2.3)	1.1 (0.5-1.3)†
Nutrients[2]			
Vitamin A (µg)	412 (282-565)	395 (198-488)	252 (96-445)*
Vitamin B_{12} (µg)	3.0 (2.8-3.4)	2.1 (1.4-3.0)	2.3 (1.5-3.4)†
Sodium (mg)	1985 (1687-2228)	1891 (1635-2245)	1445 (1199-2133)*

d) Dietary Pattern 2 below median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food group (servings per day)[1]			
Refined carbohydrates	0.9 (0.6-1.2)	1.1 (0.9-1.4)	1.5 (0.9-1.8)*
Nutrients[2]			
Vitamin E (µg)	6.5 (4.5-9.5)	5.6 (4.2-7.4)	4.2 (3.5-5.7)*
Added sugars (g)	31 (24-37)	26 (24-49)	18 (9.5-26.3)*

Data expressed as median (IQR); *p≤0.05; †p≤0.1; p.b. – post-baseline
[1]Food groups derived from food frequency questionnaire
[2]Nutrient intake derived from 3-day food diary

Table 6.12 Relative abundance of bacterial genera in feces at baseline, 6 weeks and 6 months post-baseline based on dietary patterns in children with ASD

a) Dietary Pattern 1 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacterial taxa (% of sequences)			
Erysipelotrichaceae	0.3 (0.12-0.98)	0.98 (0.22-1.23)	0.9 (0.33-1.44)†
Clostridium	0.33 (0.11-0.66)	0.11 (0.07-0.33)	0.09 (0.03-0.11)†

b) Dietary Pattern 1 below median

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacterial richness/diversity			
Shannon Index	5.7 (5.2-6.1)	5.3 (5.0-5.8)	4.8 (4.3-5.3)†
Chao 1 Index	524 (442-535)	486 (472-532)	450 (357-535)†
Bacterial taxa (% of sequences)			
Clostridium	0.23 (0.05-0.49)	0.26 (0.09-0.50)	0.05 (0.03-0.11)†
Oscillospira	0.37 (0.29-0.56)	0.15 (0.11-0.37)	0.14 (0.07-0.25)*
Dorea	0.87 (0.59-1.36)	0.54 (0.41-0.97)	0.48 (0.27-1.06)†

c) Dietary Pattern 2 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacterial taxa (% of sequences)			
Erysipelotrichaceae	1.00 (0.31-1.29)	0.77 (0.43-1.06)	0.29 (0.12-1.67)†
Clostridiaceae_Clostridium	0.23 (0.05-0.49)	0.28 (0.06-0.44)	0.05 (0.03-0.14)†
Oscillospira	0.38 (0.37-0.52)	0.28 (0.13-0.40)	0.17 (0.13-0.29) †

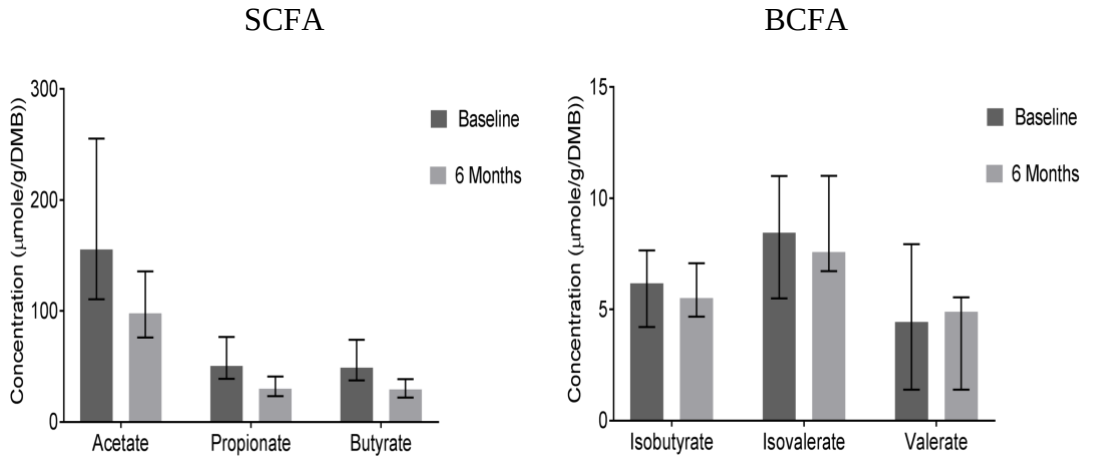
d) Dietary Pattern 2 below median

	Baseline	6-weeks p.-b.	6 months p.-b.
Bacterial taxa (% of sequences)			
Cyanobacteria	0 (0-0.007)	0.001 (0-0.005)	0.002 (0-0.02)†
Clostridiaceae_Clostridium	0.42 (0.17-0.66)	0.13 (0.08-0.39)	0.07 (0.02-0.16)†

Data expressed as median (IQR); within same group and row, significant at *p≤0.05; †p≤0.1; p.b. – post-baseline

Figure 6.9 VFA concentrations at baseline and 6-months p.-b. based on dietary pattern in children with ASD

a) Dietary pattern 1 – above median



b) Dietary Pattern 1 – Below Median

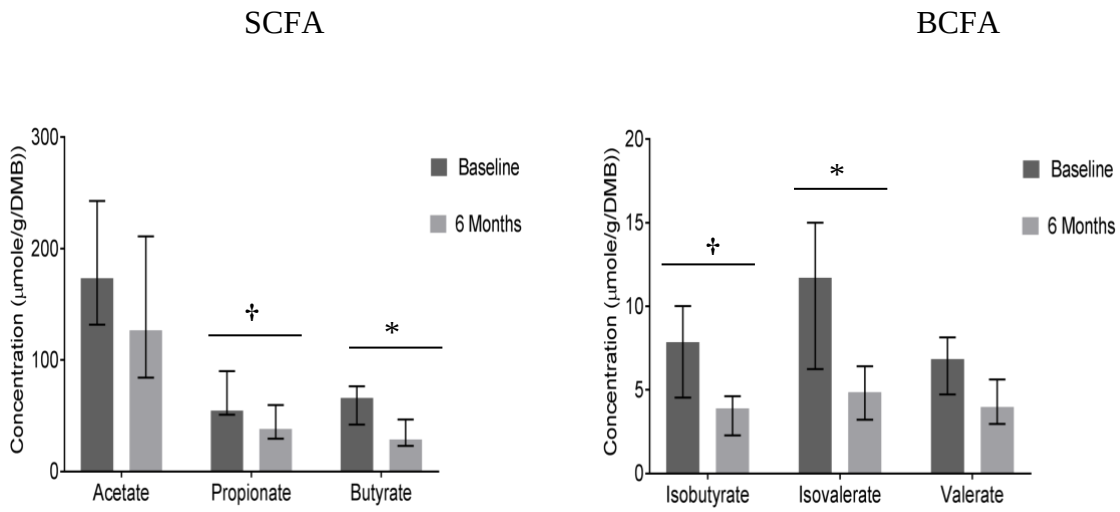
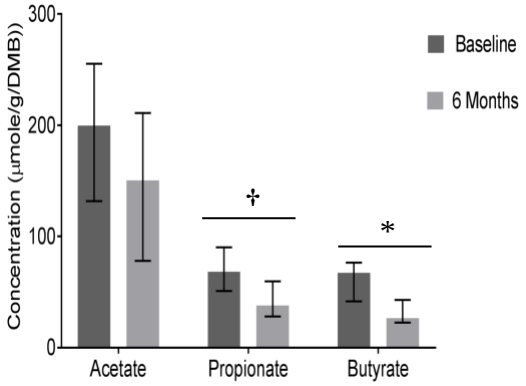


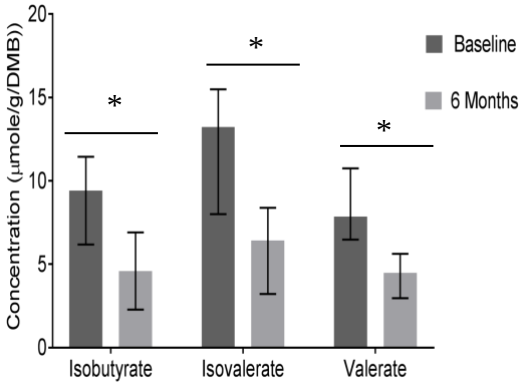
Figure 6.9 (cont.)

c) Dietary Pattern 2 – Above Median

SCFA

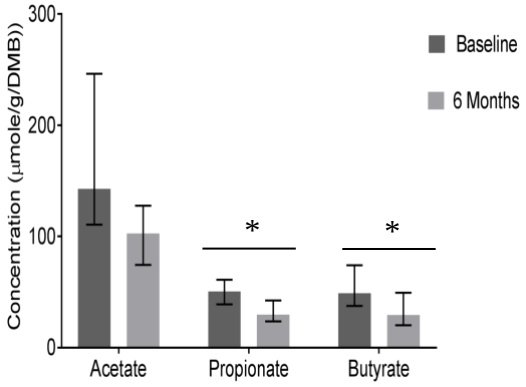


BCFA

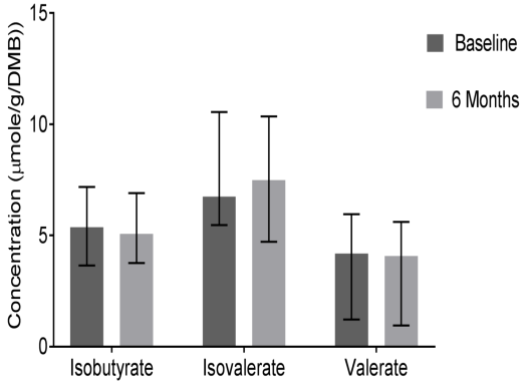


d) Dietary Pattern 2 – Below Median

SCFA



BCFA



Median (IQR); *p≤0.05; †p≤0.1; Dietary patterns were derived from food frequency questionnaire and principal component analysis (Chapter 5); CONT, unaffected controls; ASD, Autism Spectrum Disorder; DMB, dry matter basis; SCFA, short chain fatty acids; BCFA, branch chain fatty acids; p.-b., post-baseline

Table 6.13 Food group and nutrient intake at baseline, 6 weeks post-baseline and 6 months post-baseline based on dietary pattern in CONT children

a) Dietary pattern 1 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food Group (serving per day)[1]			
Protein	0.9 (0.5-1.7)	1.9 (1.2-2.6)	1.5 (1.1-2.2)*
Snacks	0.9 (0.6-1.5)	0.7 (0.5-1.2)	0.5 (0.3-0.6)†
Nutrient[2]			
Protein (g)	48 (34-56)	53 (47-74)	58 (42-63)†
Vitamin B12 (µg)	2.7 (2.0-3.4)	3.2 (2.1-5.1)	3.6 (3.1-4.4)†
Sodium (mg)	1673 (1380-2458)	2109 (1549-3352)	2044 (1730-2919)*
Manganese (mg)	2.4 (1.9-3.5)	2.0 (1.4-3.3)	1.9 (1.4-2.6)*
Added sugars (g)	40 (28-44)	31 (26-41)	24 (15-36)*
Whole grains (g)	0.7 (0.4-1.7)	0.5 (0.3-1.2)	0.4 (0.09-1.2)†

b) Dietary pattern 1 below median.

	Baseline	6-weeks p.-b.	6 months p.-b.
Food Group (serving per day)[1]			
Fruit	1.9 (1.4-2.2)	1.8 (1.0-2.3)	2.5 (2.1-2.6)*
Grains	0.6 (0.5-1.0)	0.7 (0.5-0.99)	0.8 (0.6-1.0)†
Nutrient[2]			
Vitamin D (µg)	4.99 (3.8-6.4)	4.3 (2.5-6.5)	2.5 (2.2-4.4)*
Riboflavin (µg)	1.7 (1.4-1.9)	1.6 (1.3-1.9)	1.3 (1.2-1.6)*
Vitamin B12 (µg)	3.5 (3.2-5.1)	3.5 (2.2-4.4)	3.1 (2.1-3.6)†
Selenium (µg)	74 (67-87)	84 (74-97)	65 (60-88)†

Table 6.13 (cont.)

c) Dietary Pattern 2 above median

	Baseline	6-weeks p.-b.	6 months p.-b.
Nutrients[2]			
Vitamin K (μg)	51 (38-73)	51 (33-77)	34 (25-47)*
Vitamin C (μg)	67 (48-131)	53 (29-74)	37 (21-54)*
Added sugars (g)	33 (27-44)	28 (17-40)	27 (15-50)†

d) Dietary Pattern 2 below median

	Baseline	6-weeks p.-b.	6 months p.-b.
Food group (servings per day[1]			
Fruit	1.7 (1.3-2.0)	1.8 (1.4-2.9)	2.5 (2.1-3.0)*
Legumes, nuts, and seeds	0.3 (0.2-0.7)	0.3 (0.2-0.9)	0.2 (0.16-0.28)*
Refined carbohydrates	0.8 (0.5-1.2)	0.7 (0.6-0.9)	1.2 (0.2-1.9)†
Protein	0.8 (0.6-1.3)	1.2 (0.9-2.1)	1.3 (1.2-1.7)*
Nutrients[2]			
Total fat (g)	51 (42-56)	54 (48-71)	60 (46-68)*
SFA (g)	17 (13-22)	19 (15-28)	21 (14-31)*
Vitamin K (μg)	36 (23-50)	42 (25-81)	54 (37-73)†
Whole grains (g)	1.6 (0.8-2.6)	0.8 (0.3-1.4)	0.9 (0.5-1.2)*

Data expressed as Median (IQR); *p≤0.05; †p≤0.1; p.b. – post-baseline;
[1]Food groups derived from food frequency questionnaire
[2]Nutrient intake derived from 3-day food diary

Table 6.14 Relative abundance of bacterial genera in feces at baseline, 6 weeks and 6 months post-baseline based on dietary patterns in CONT children

a) Dietary Pattern 1 above median

Bacterial taxa (% of sequences)	Baseline	6-weeks p.-b.	6 months p.-b.
Parabacteroides	1.8 (0.7-3.4)	1.2 (0.6-2.0)	1.0 (0.2-1.5)†

b) Dietary Pattern 1 below median

Bacterial taxa (% of sequences)	Baseline	6-weeks p.-b.	6 months p.-b.
Butyrivibrio	0.02 (0.001-0.07)	0.02 (0.004-0.05)	0.01 (0.002-0.02)*
Veillonella	0.02 (0.01-0.05)	0.01 (0.001-0.04)	0.004 (0.001-0.03)*

c) Dietary Pattern 2 above median

Bacterial taxa (% of sequences)	Baseline	6-weeks p.-b.	6 months p.-b.
Proteobacteria	0.84 (0.41-1.74)	0.59 (0.16-1.91)	0.36 (0.22-1.06)*
Slackia	0 (0-0)	0 (0-0)	0.001 (0-0.002)†

Data expressed as median (IQR); within same group and row, significant at *p≤0.05; †p≤0.1; p.b. – post-baseline

Table 6.15 Nutrient intake at baseline, 6-weeks and 6-months post baseline in children with moderate ASD symptoms

Nutrient intake[1]	Baseline	6-weeks p.-b.	6 months p.-b.
Vitamin A (µg)	473 (277-651)	331 (225-502)	252 (160-306)*
Vitamin B12 (mg)	2.9 (2.2-3.5)	2.6 (1.6-3.2)	2.3 (1.5-2.6)†
Sodium (g)	1987 (1635-2261)	1606 (1313-2179)	1445 (1272-2125)†
Added sugars (g)	36 (26-44)	31 (17-45)	17 (13-24)†

Data expressed as median (IQR); *p≤0.05; †p≤0.1 in same group and row significant;
[1]nutrient intake derived from 3-day food diary
ASD, Autism spectrum Disorder; SOCDEF, social deficit score

Table 6.16 Microbiota composition and VFA concentration in children with ASD at baseline, 6-week and 6-month post-baseline based on severity of SOCDEF symptoms

a) Microbiota and VFA changes in children with moderate social deficit symptoms

	Baseline	**6-weeks p.-b.**	**6 months p.-b.**
Bacteria (relative abundance)			
Staphylococcus	0.009 (0.004-0.02)	0.02 (0.006-0.07)	0.007 (0.002-0.09)†
Clostridium	0.38 (0.11-0.68)	0.14 (0.07-0.46)	0.05 (0.02-0.14)*
VFA (µmole/g (DMB))			
Propionate	53 (38-82)	.	36 (24-42)*
Butyrate	61 (37-73)	.	23 (22-31)*
Isobutyrate	7.2 (5.5-10.0)	.	4.6 (2.4-5.9)*
Isovalerate	11 (6.4-15.0)	.	6.4 (3.96-7.6)*

b) Microbiota and VFA changes in children with severe social deficit symptoms

	Baseline	**6-weeks p.-b.**	**6 months p.-b.**
Bacteria (relative abundance)			
Clostridiaceae	0.97 (0.51-3.74)	1.62 (0.51-3.82)	0.35 (0.3-0.38)†
Methanobrevibacter	0.0007 (0.0007-0.002)	0 (0-0.001)	0 (0-0.009)*
VFA (µmole/g (DMB))			
Acetate	253 (134-344)	.	106 (92-127)*
Propionate	56 (51-84)	.	30 (26-38)*
Butyrate	75 (64-80)	.	41 (20-50)*

Data expressed as median (IQR); *p≤0.05; †p≤0.1 in same group and row significant; VFA, volatile fatty acids

CHAPTER 7
Summary and Future Directions

This research study aimed to further our understanding of the potential importance of dietary intake on the microbiota-brain connection in children with ASD. Previously published research has demonstrated that the GI microbiota differs between children with ASD and unaffected controls, that bacterial taxa can influence some symptoms of ASD and that dietary intake is often challenging in children with ASD. However, prior to this research no one study had combined these individual measurements in one research project. Here, for the first time, we demonstrate the impact of diet on the microbiota as well as long-term variability patterns in children with ASD.

To first investigate whether the ASD population of this study also harbors a "dysbiotic" microbiome that could potentially impact some symptoms of ASD, the first part of this research project investigated microbiota composition and microbial metabolites using16S rRNA sequencing, quantitative Polymerase Chain Reaction and gas chromatography (Chapter 4). In support of our hypothesis and consistent with the literature, the microbiota composition of children with ASD differed from unaffected controls and higher concentrations of volatile fatty acids (VFAs) were noted. Specifically, higher abundances of Clostridiaceae, Clostridiales and Clostridium were observed in children with ASD, which are bacterial taxa of high interest in ASD symptomology. Likewise, Peptostreptococaceae and *Faecalibacterium* were two bacterial taxa identify to positively predict social deficit scores.

Next, the impact of dietary factors on the GI microbiota composition and potential three-way interactions between diet-microbiota-ASD symptoms were investigated using correlation analysis, dietary pattern analysis, association with feeding problems (i.e., picky eating) and

moderation analysis (Chapter 5). First, we found that bacteria shown to differ between the groups (e.g., Clostridiales) and those positively predicting social deficit scores (i.e., *Faecalibacterium*) showed strong correlations with food (e.g., fruit, fried food) and nutrient (e.g., insoluble dietary fiber) intake, suggesting that dietary intake could contribute to the microbial differences observed in children with ASD. Second, feeding problems often observed in children with ASD (e.g., picky eating or repetitive eating pattern) were associated with unique microbial abundances and VFA concentrations. And third, in support of our hypothesis, a dietary pattern characterized by high intakes of healthy foods such as fruits, vegetables, legumes, nuts and seeds and grains was associated with microbial taxa known to exhibit beneficial effects on the host. On the other hand, an unhealthier dietary pattern defined by high intakes of fried food, kid's meals, snacks and protein food harbored a microbial profile that was represented by potentially harmful bacteria and also demonstrated higher concentrations of VFAs. Contrary to our hypothesis, diet-induced microbial profiles showed no relationship with social deficit symptoms, but associations with GI symptoms were observed. This lack of association might be due to the number of children included in the study or the ASD symptoms measured. Previous studies have reported that the GI microbiota might be more strongly correlated with other symptoms of ASD (i.e., restricted and repetitive behaviors).

Lastly, longitudinal samples were analyzed in order to investigate temporal microbial variability and the impact of diet on microbiota stability in children with ASD (Chapter 6). Confirming our hypothesis we found that dietary measures were associated with long-term stability of the microbiota and that an unhealthier dietary pattern showed greater variability in microbial structure. However, contrary to our hypothesis, the microbial profile of children with ASD was not more variable when compared to unaffected controls, but both group exhibited

unique stabilizing and destabilizing microbial taxa. Also discordant to our hypothesis we found that less variability in the presence and absence of OTUs was correlated with more severe social deficit symptoms in children with ASD. This observation could potentially attributed to the constant presence of bacteria hypothesized to influence ASD symptomology (i.e., *Clostridium*).

Taken together, the results presented herein demonstrate that the GI microbiota composition as well as microbial stability is strongly influenced by dietary intake in children with ASD. Thus, future research investigating the microbiota composition of children with ASD should consider collecting information on dietary intake to delineate whether the microbial differences observed in children with ASD are due to dietary factors or are inherent to ASD itself. The data presented in this dissertation can serve as preliminary evidence for several future research studies. Overall, studies using larger sample sizes are required to define an "ASD microbiome" and to provide sufficient power to demonstrate the importance in considering dietary intake when analyzing the GI microbiota composition.

First, studies measuring other symptoms of ASD (e.g., restricted/repetitive behaviors) and potentially using better tools to measure ASD symptoms (e.g., Autism Diagnostic Interview – Revised or ADOS) are needed to further understand whether diet can be a moderator of the microbiota-brain connection. Due to the young age of the children in this cohort, the PDDBI-SV was the best validated tool available to quantify ASD symptoms in children under the age of 4. While this tool can be used in research (Cohen, 2011), it is limited by the ability to only measure social deficit symptoms. Previous studies have indicated that restricted and repetitive behaviors might be more influenced by the microbiota composition (Tomova et al., 2015). Measuring other symptoms of ASD and utilizing similar analytic approaches to the ones described herein can

provide more important information on the diet-microbiota-ASD symptom connection and could provide further evidence to potentially use diet as a managing strategy for ASD symptoms.

Second, future research should also focus on further investigating the microbial stability in children with ASD and determine whether microbial changes can be linked to symptom changes. Although previous research has indicated that microbial stability is more beneficial (Bäckhed et al., 2012), herein we observed the opposite relationship, indicating that children with ASD and less microbiome variability had higher social deficit scores. Assessing whether this relationship can be observed in other cohorts is important to determine whether it is of importance in ASD symptomology. Future studies collecting samples more frequently could provide more valuable evidence. Likewise, previous research has shown that the onset of ASD symptoms could be associated with GI disturbance and higher levels of Clostridiales (Williams et al., 2011). Thus, future studies investigating the microbiota of at-risk children (i.e., siblings of children with ASD) could delineate whether changes in the microbiota composition could contribute to the development of ASD symptoms.

Third, associations between feeding behavior (e.g., picky eating, repetitive eating patterns, specialty diet) and microbiota composition and variability were based on single item questions that were answered by the parents. Thus, these associations hold some bias due to the nature of the questions. Future studies using home observations or detailed questionnaires (e.g., Brief Autism Mealtime Behavior Inventory (Lukens and Linscheid, 2008) to assess foods included in the diet will provide more unbiased information on the impact of exhibited feeding problems on the microbiota.

Finally, due to the observational nature of this dissertation, it was not possible to investigate a cause-and-effect relationship between ASD and the microbiota. Therefore, future

studies are needed to investigate underlying mechanisms of the microbiota-brain axis in children with ASD and delineate how the higher abundance of some bacteria could potentially influence symptomology. Thereby, gnotobiotic animal models could be of fundamental importance. Likewise, randomized controlled trials investigating the potential of dietary interventions in the management of some symptoms of ASD should collect fecal samples in order to investigate whether the GI microbiota could be a potential link between diet and symptoms. Studies have proposed that the GI microbiota could be a key mediator in the diet-brain connection (Dawson et al., 2016) and animal models have demonstrated that diet-induced changes in the GI microbiota could result in behavioral changes (Li et al., 2009, Pyndt Jørgensen et al., 2014). Expanding these research efforts into the ASD population could provide valuable evidence for potential new therapeutic avenues.

Chapter 8

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APPENDIX A.

Differences in Microbiota, VFA and Nutrition Between Children with ASD and Their Siblings

A.1 Introduction.

The gastrointestinal (GI) microbiota composition is influenced by various environmental (i.e., diet, living environment) as well as genetic factors. Thus, including siblings as a control group when analyzing the microbiota in disease states is an attractive option in order to control for as many variables as possible that could potentially impact the GI microbiota. Previous studies investigating the microbiota composition in children with ASD that included a group of siblings as a control group showed that siblings often fall between children with ASD and unrelated controls in the microbiota composition (Finegold et al., 2010; Tomova et al., 2015). Therefore, we recruited siblings as control subjects for the current study. Initially, 15 sibling controls were recruited, but baseline samples were only collected from 7 of them. Due to the small sample size, the siblings were not included as a group in the overall analysis. Thus, the comparison between siblings and children with ASD was done separately and is reported in this Appendix.

A.2 Methods

All samples were collected and analyzed as previously described in Chapter 4 and 5. Children with ASD were matched with their perspective siblings. For some children with ASD, more than one sibling was enrolled in the study. Thus, the sample size for this analysis is n=5 for children with ASD (ASD) and n=7 for sibling controls (SIB).

A.3 Results

Demographics

The demographics for each group are shown in **Table A.1**. Children with ASD were slightly older (4.4±1.7 years) vs. than their siblings (3.5±1.9 years); however, there was no statistical difference between the groups. Children with ASD were slightly heavier (p=0.09) and taller (p=0.08) compared to their siblings, but the mean BMI did not differ between the groups. No significant difference between nutritional supplement use, route of birth, gestational age or early feeding mode. Frequent antibiotic use was reported in one child with ASD but in none of the siblings (p=0.003). Regarding eating behavior, children with ASD were less likely (p=0.05) to include 20 or more foods in their diet and were more likely to have a repetitive eating pattern (p=0.008) compared to their siblings. No difference was observed regarding picky eating behavior.

GI Severity and Stool Consistency

Gastrointestinal characteristics are shown in **Table A.2**. Overall the GI severity score did not differ between the groups. Only abdominal pain scores were higher (p=0.03) in children with ASD compared to their siblings. Additionally, stool consistency as measured by the Bristol stool chart did not differ between the groups.

Microbiota Composition

α- diversity (Chao 1 Index, Shannon, Index, Simpson Index, observed OTUs) and β-diversity did not differ between the groups (**Table A.3**).

Abundances of individual bacterial taxa are shown in **Table A.4**. No differences at the phyla, order or family level were detected between the groups. On the genera level, children with ASD tended to have higher abundance of *Leuconostoc* (p=0.07) and *Sutterella* (p=0.06), but lower abundance of *Oscillospira* (p=0.06) and *Coprobacillus* (p=0.07).

VFA Concentration

Fecal VFA concentrations did not differ between the groups (**Figure A.1**).

Nutrient intake

Levels of nutrient intake derived from the 3-day food record is shown in **Table A.5**. Children with ASD had higher intakes of energy (p=0.09), total fat (p=0.01), MUFA (p=0.01) and PUFA (p=0.01) compared to their siblings.

Servings of Food Groups

Intakes of servings of food groups in both groups are shown in **Table A.6**. Siblings tended to have higher intakes of starchy foods (p=0.07) as well as Kid's meals (p=0.07) compared with children with ASD.

A.4 Discussion

There is increasing interest to study the siblings of children with ASD to study potential early biomarkers (biochemical and behaviorally) of ASD in a genetically at-risk population. When studying the microbiota composition, including subjects living in the same household and having similar genetics can provide important points of control. Previous studies have shown that

genetics (Hallmayer et al., 2011) impacts the GI microbiota. Additionally, families tended to have more similar microbiota structures compared to unrelated subjects (Song et al., 2013). Various studies have shown that the GI microbiota composition of children with ASD is more similar to their siblings compared to unrelated controls (Tomova et al., 2015; Wang et al., 2011) This could be due to similar diet, living environment or transmission of bacteria between siblings (Finegold et al., 2010).

Here, we observed that the microbiota composition was more similar between children with ASD and their siblings compared to unaffected controls (Chapter 4). Likewise, no differences were observed in concentration of VFAs. Although siblings were less likely to include more than 20 foods in their diet and were more likely to exhibit repetitive eating patterns, only minimal differences were noted regarding the food and nutrient intake. These minimal differences in the intake of food could be attributed to the fact that siblings are most likely offered similar foods at the home. Interestingly, abdominal pain scores were higher in children with ASD, suggesting that the GI tract might be important in ASD symptomology.

These results illustrate that using a sibling control group can be important in controlling for factors such as living environment and genetics. Likewise, studying the microbiota of siblings can yield interesting new information on how the microbiota can potentially influence ASD development. Longitudinal studies including younger siblings of children with ASD would be interesting to elucidate whether the microbiota is dysbiotic in children that are more likely to develop ASD.

A.5 Tables and Figures

Table A. 1 Demographic characteristics of all study participants

Characteristic	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Age (years)	4.4±1.7	3.5±1.9	NS
Gender (n)			
Male	5	5	
Female	0	2	
Weight (kg)	20±4.3	13.8±4.7	0.09
Height (meters)	1.09±0.07	0.94±0.13	0.08
Mean BMI and Percentile (BMI-for-age)			
Male			
Female			
Nutritional Supplement use (n (%))			NS
Yes	3	5	
No	2	2	
Route of Birth (n (%))			NS
Vaginal	2	5	
Planned C-section	0	0	
Emergency C-section	2	0	
Gestational age (n (%))			NS
<37 weeks	1	1	
37-42 weeks	4	6	
>42 weeks	0	0	
Early Feeding Mode (n (%))			NS
Breast-fed only	1	3	
Breast-fed in combination with formula	2	4	
Formula only	2	0	
Antibiotics use in early life (n (%))			0.003
Yes	1	0	
No	4	7	
Picky Eater			NS
Yes	0	0	
No	5	7	
More than 20 foods in diet (n (%))			0.05
Yes	3	7	
No	2	0	
Repetitive eating pattern			0.008
Yes	5	0	
No	0	7	

Data expressed as mean±SD or n
ASD, Autism spectrum disorder, SIB=Siblings

Table A.2 Differences in GI Severity scores and stool consistency between children with ASD (ASD) and siblings

Characteristic	**ASD (n=5)**	**CONT (n=7)**	**p-value**
GI Severity Score[1]	1.2±1.3	1.3±1.9	NS
Constipation	0.6±0.9	1±0.8	NS
Diarrhea	0±0	0±0	NS
Stool Smell	0.6±0.5	0.1±0.4	NS
Flatulence	0.4±0.9	0.4±0.8	NS
Abdominal Pain	0.6±0.5	0±0	0.03
Stool Consistency (n)[2]			NS
Type 1 (separate hard lumps)	0	1	
Type 2 (sausage shaped but lumpy)	0	1	
Type 3 (sausage-shaped with cracks on surface)	0	2	
Type 4 (smooth and soft)	3	3	
Type 5 (soft blobs)	1	0	
Type 6 (mushy)	1	0	
Type 7 (watery)	0	0	

Data expressed as mean ± SD or n

[1]GI severity scores were derived from the GI severity index (possible range 0-7)

[2]Stool consistency was measured using the Bristol Stool chart

Table A.3 α-Diversity measures between children with ASD (ASD) and siblings (SIB)

Diversity/Richness Measure	ASD (n=5)	SIB (n=7)	p-value
Shannon Index	5.6±0.3	5.7±0.5	NS
Simpson Index	0.9±0.02	1±0.01	NS
Observed OTUs	523±42	534±61	NS
Chao 1 Index	584±39	571±51	NS

Data expressed as mean ± SD;
ASD, Autism spectrum disorder; SIB, sibling group

Table A.4 Relative abundances of bacterial taxa detected in feces of children with ASD (ASD) and siblings (SIB).

Phyla	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Euryarchaeota	0.02±0.03	0.02±0.05	NS
Actinobacteria	7.15±8.2	8.48±7.1	NS
Bacteroidetes	42.96±19.1	25.80±13.9	NS
Cyanobacteria	0.00±0.00	0.01±0.02	NS
Firmicutes	45.40±18	62.15±22.1	NS
Fusobacteria	0.00±0	0.13±0.34	NS
Proteobacteria	0.76±0.75	1.63±3.9	NS
Tenericutes	0.00±0.00	0.61±1.6	NS
Verrucomicrobia	3.71±7.6	0.02±2.3	NS

Order	ASD w/ SIB(n=5)	SIB (n=7)	p-value
Bacteroidales	0.00±0.00	0.00±0.00	NS
Streptophyta	0.003±0.006	0.01±0.02	NS
Clostridiales	6.3±2.2	4.9±2.7	NS
RF32	0.04±0.09	0.003±0.007	NS
RF39	0.00±0.00	0.6±1.6	NS

Family	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Coriobacteriaceae	0.04±0.04	0.04±0.06	NS
RF16	0.01±.03	0.01±0.03	NS
Rikenellaceae	2±2.1	2.9±4.1	NS
S24-7	0±0	0±0	NS
Barnesiellaceae	0.7±1.5	0.5±1.2	NS
Christensenellaceae	0.02±0.04	0.0006±0.005	NS
Clostridiaceae	1.5±1.4	1.7±1.7	NS
EtOH8	0±0	0±0	NS
Lachnospiraceae	11.7±3.9	14±7.1	NS
Peptostreptococcaceae	0.06±0.06	0.12±0.13	NS
Mogibacteriaceae	0.01±0.01	0.007±0.02	NS
Erysipelotrichaceae	0.3±0.4	1.12±1.3	NS
Enterobacteriaceae	0.6±0.8	1.5±3.8	NS

Genera	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Archea – Euryarchaeota			
Methanobrevibacter	0.02±0.03	0.02±0.05	NS
Actinobacteria			
Bifidobacterium	9.8±0.09	10.1±0.05	NS
Adlercreutzia	0.01±0.01	0.02±0.03	NS
Collinsella	2.2±3.2	1.3±1.8	NS
Eggerthella	0.05±0.04	0.15±0.16	NS
Slackia	0.0008±0.0007	0.001±0.002	NS
Bacteroidetes			

Table A.4 (cont.)

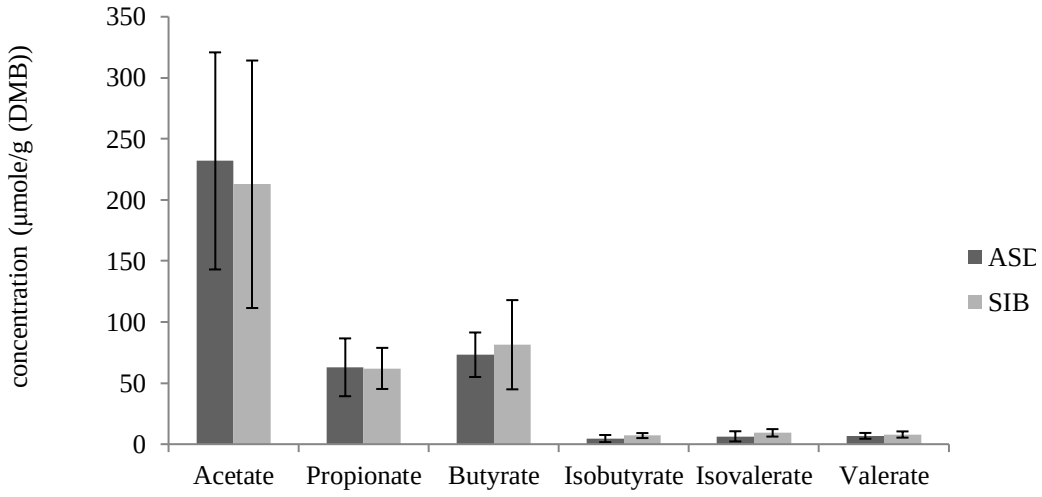
Genera	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Bacteroides	23.1±16.1	17.1±3.9	NS
Parabacteroides	1.1±1.1	3.6±6.9	NS
Prevotella	11±0.6	10.2±0.8	NS
Alistipes	0.02±0.05	0.2±0.5	NS
Butyricimonas	0.03±0.07	0.01±0.03	NS
Odoribacter	0.07±0.09	0.07±.05	NS
Paraprevotella	0.0003±0.0006	0.0005±0.0005	NS
Prevotella	0.07±0.2	0.02±0.05	NS
Firmicutes			
Staphylococcus	0.07±0.1	0.08±0.08	NS
Enterococcus	0.04±0.09	0.0002±0.0003	NS
Lactobacillus	7.1±0.6	7.1±0.9	NS
Leuconostoc	0.0004±0.0005	0±0	0.07
Lactococcus	0.03±0.06	0.05±0.1	NS
Streptococcus	0.9±1.3	3.9±5.1	NS
Turicibacter	0.09±0.12	0.06±0.03	NS
02d06	0.01±0.009	0.01±0.02	NS
Clostridaceae_Clostridium	1.00±1.4	0.55±0.8	NS
SMB53	2.11±1.6	4.18±4.4	NS
Sarcina	0.05±0.1	0.00±0.002	NS
Eubacterium	0.007±0.008	0.2±.6	NS
Anaerostipes	0.18±0.2	0.05±0.03	NS
Blautia	2.22±1.5	1.70±1	NS
Butyrivibrio	0.03±0.01	0.02±0.0007	NS
Coprococcus	4.94±3.3	5.02±1.3	NS
Dorea	0.83±0.5	1.43±0.9	NS
Lachnospira	0.19±0.3	0.07±0.08	NS
Roseburia	0.46±0.6	1.26±0.1.4	NS
Peptococcus	0±0	0±0	NS
Anaerotruncus	0.005±0.007	0.03±0.03	NS
Peptostreptococcaceae_ Clostridium	0.0002±0.0005	0.0003±0.0003	NS
Faecalibacterium	11.80±9.8	6.94±7.2	NS
Oscillospira	0.25±0.2	0.57±0.4	0.06
Ruminococcus	3.21±3.4	2.16±1.5	NS
Acidaminococcus	0.04±0.09	0.02±0.06	NS
Dialister	0.98±1.3	2.47±6.0	NS
Megamonas	0.00±0.00	0.00±0.00	NS
Megasphaera	0.00±0.00	0.00±0.00	NS
Phascolarctobacterium	0.13±0.27	0.07±0.14	NS
Succiniclasticum	0.00±0.00	0.00±0.00	NS
Veillonella	0.14±0.26	0.06±0.05	NS
Catenibacterium	0.00±0.00	0.00±0.00	NS
Coprobacillus	0.00±0.00	0.01±0.009	0.07

Table A.4 (cont.)

Genera	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Holdemania	0.01±0.007	0.01±0.01	NS
Fusobacteria			
Fusobacterium	0±0	0.13±0.3	NS
Proteobacteria			
Sutterella	0.3±0.4	0.04±0.04	0.06
Bilophila	0.03±0.06	0.03±0.03	NS
Campylobacter	0.00±0.00	0.00±0.00	NS
Haemophilus	0.74±1.5	0.03±.03	NS
Verrucomicrobia			
Akkermansia	0.26±0.4	1.17±2.3	NS

Data expressed as mean ± SD

Figure A.1 VFA concentrations did not differ between SIB and ASD



Mean ± SD
SIB, siblings; ASD, Autism Spectrum Disorder; DMB, dry matter basis

Table A.5 Comparison of Nutrient Intake between children with ASD (ASD) and their siblings (SIB).

Variable	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Macronutrients:			
Energy (kcal)	1487±455	1063±338	0.09
Total Fat (g)	64±23	39±9	0.08
Omega-3 Fatty Acids (g)	1.1±0.4	0.7±0.2	0.07
SFA (g)	22±11	14±4	NS
MUFA (g)	22±7	13±3	0.01
PUFA (g)	15±4	8.9±2.5	0.01
Total Carbohydrate (g)	191±57	147±67	NS
Total Sugars (g)			NS
Added Sugars (g)	45±25	34±21	NS
Total Grains (oz equivalents)	7.1±2.8	5.8±2.4	NS
Whole Grains (oz equivalents)	0.8±1.3	0.8±1.2	NS
Refined Grains (oz equivalents)	6.2±3	5±1.8	NS
Total Protein (g)	42±17	34±8.4	NS
Dietary Fiber			
Total Dietary Fiber (g)	11.7±5	8.0±4.7	NS
Soluble Dietary Fiber (g)	4.3±1.2	3.1±1.6	NS
Insoluble Dietary Fiber (g)	7.4±4.3	4.7±3.1	NS
Pectin (g)	1.3±0.9	0.6±0.6	NS
Vitamins			
Vitamin A (μg)	538±307	363±177	NS
Vitamin D (μg)	2.1±1.7	3.4±3.2	NS
Vitamin E (mg)	6.3±2.2	4.5±1.8	NS
Vitamin K (μg)	39±21	23±12	NS
Vitamin C (mg)	25±18	23±15	NS
Thiamin (mg)	1.4±0.4	1.2±0.5	NS
Riboflavin (mg)	1.6±0.7	1.4±0.8	NS
Niacin (mg)	17±5.8	13±7.4	NS
Pantothenic Acid (mg)	2.8±1.4	1.9±1	NS
Vitamin B_6 (mg)	1.5±0.7	1.1±0.8	NS
Folate (μg)	310±247	330±333	NS
Vitamin B_{12} (μg)	3.3±2	2.5±1.7	NS
Minerals			
Calcium (mg)	737±282	675±29	NS
Phosphorus (mg)	858±269	659±241	NS
Magnesium (mg)	155±78	115±65	NS
Iron(mg)	13 ±8.6	12.5±12.6	NS
Zinc (mg)	6.4±5.3	6.7±5.4	NS
Copper (mg)	0.6±0.3	0.44±0.2	NS
Selenium (mg)	64±25	53±15	NS
Sodium (mg)	2028±286	1711±260	NS
Potassium (mg)	1290±259	886±535	NS

Table A.5 (cont.)

Variable	ASD w/ SIB (n=5)	SIB (n=7)	p-value
Manganese (mg)	1.9±1	1.7±1.10	NS

Data expressed as mean ± SD; nutrient intake derived from 3 day food records
ASD, autism Spectrum disorder group; SIB, sibling group; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

Table A.6 Differences in intake of food groups between children with ASD (ASD) and their siblings (SIB)

Food Group	**ASD w/ SIB (n=5)**	**SIB (n=7)**	**p-value**
Fruit	1.9±1.2	2.7±1.3	NS
Vegetables	1.2±0.9	1.7±1.1	NS
Legumes	0.3±0.2	0.2±0.1	NS
Starchy Foods	0.2±0.1	0.4±0.3	0.07
Starchy Vegetables	0.5±0.4	0.5±0.3	NS
Juice	0.6±0.1	0.5±0.4	NS
Sweetened Beverages	0.5±0.1	0.4±0.4	NS
Grains	0.7±0.1	0.8±0.8	NS
Refined Carbohydrates	1.2±0.9	1.2±0.5	NS
Fried Foods	0.2±0.1	0.2±0.1	NS
Protein	1.4±0.3	1.6±0.8	NS
Dairy	3.8±1.2	3.7±2.2	NS
Snack	1.3±0.8	1.1±0.6	NS
Sweets	2.9±1.8	1.8±1.2	NS
Kid's Meal	0.7±0.2	1.3±0.5	0.07
Fish	0.1±0.1	0.1±0.1	NS
Condiments	0.4±0.1	0.3±0.2	NS

Data expressed as mean ± SD; Food group intakes were derived from YAQ

APPENDIX B.

YAQ Dietary Pattern Groupings

Food Group	Foods included
Fruit	Grapes, Bananas, Strawberries, Blueberries, Peaches, Plums, Apricots, Apples, Applesauce, Pears, Oranges, Grapefruit, Watermelon, Pineapple, Cantaloupe, Melon
Vegetables	Green Beans, Broccoli, Cauliflower, Mixed Vegetables, Spinach, Collard Greens, Kale, Cooked Spinach, Bell Peppers, Zucchini, Summer Squash, Eggplant, Carrots, Celery, Tomatoes, Lettuce, Tossed Salad, Coleslaw, Cabbage, Okra
Legumes, Nuts and Seeds	Beans, Lentils, Peanuts, Other Nuts, Mixed Dried Fruit Trail Mix, Seeds
Starchy Vegetables	Corn, Peas, Potatoes, Yams, Sweet Potatoes
Starchy Foods	Lasagna, Baked Ziti, Ravioli, Spaghetti or Pasta with Tomato Sauce, Noodles or Pasta (plain), Pasta Salad, Potato Salad,
Juice	Fruit Juice, Tomato Juice, V8 fusion
Sweetened non-dairy beverages	Diet Soda/Pop, Soda/Pop, Iced tea, Sugar Free or Low Calorie Drinks, Energy Drinks
Grains	Oatmeal, Whole Wheat Bread, Brown Rice
Refined Carbohydrates	White Bread Pita Bread, English Muffins, Bagels, Rolls, Muffin or Cornbread, Croissant, Biscuit, White Rice, Pancakes, Waffles, Corn or Flour Tortilla
Fried Foods	French Toast, French Fries, Tater Tots, Hash Browns
Protein Foods	Chicken, Turkey, Beef, Pork, Lamb, Meatballs, Salami, Bologna, Tuna, Sausage, Eggs, Bacon, Soy Dishes, Liver
Dairy	Yogurt, Butter, Margarine, Whipped Cream, Milk, Cheese, Cream Cheese, Milkshakes, Breakfast Drinks, Cottage and Ricotta Cheese, Frozen Yogurt, Ice Cream
Snacks	Pretzels, Graham Crackers, Crackers, Potato Chips, Corn Chips, Doritos, Snack Bars, Energy Bars, Protein Bars, Popcorn
Sweets	Fruit Snack, Cake, Cupcake, Pie, Pudding, Jello, Popsicle, Jams, Jellies, Syrup, Honey, Snack Cakes, Donuts, Cookies, Brownies, Chocolate, Other Candy Bars, Other Candy without Chocolate, Poptarts, Danish, Cinnamon Rolls, Pastry, Peanut Butter and Jelly Snacks
Kids Meals	Chicken Nuggets, Fish Sticks, Hamburger. Hot Dogs, Macaroni and Cheese, Pizza, Burritos, Tacos, Veggieburger, Cheeseburger, Grilled Cheese Sandwich
Fish	Fish, Shrimp, Lobster, Scallops
Condiments	Ketchup, Mayonnaise, Salad Dressing, Salsa