Gut and neurodevelopment

The role of gut microbiota dysbiosis in attention-deficit hyperactivity disorder and autism spectrum disorder

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CV

I grew up in the village of Nøvling, just outside of Aalborg in Northern Denmark, and graduated from the Hasseris Gymnasium high school. While I always had a fascination with the natural world during childhood, a teacher at this high school managed to fuel my passion for how even minute effects or functions are associated with several highly complex processes in the body.

Following a gap year in Malta, in 2008, I began my studies for a bachelor's degree in medicine with an Industrial Specialization at Aalborg University. This originated from a conviction that a basic understanding of the clinic was essential for a proper understanding of the implications of human biology. Based on the same idea, I started volunteering as a first aider for the Red Cross at the same time. In 2013, I graduated from Aalborg University with a master's degree in Medicine with an Industrial Specialization, and a focus on Biomedicine. This combination of biomedical studies, an understanding of the clinic, and interaction with patients in distress led me to look for jobs at the hospital. This soon landed me a job at the Centre for Clinical Research, North Denmark Regional Hospital under Suzette Sørensen and Ulrik Baandrup.

During the next four years, I was involved in several different projects and I slowly warmed to the idea of initiating a Ph.D. project myself. Therefore, when Suzette and Søren suggested this project, I had no hesitation in accepting it.

PREFACE

To modern ears, the clinical use of fecal matter may sound strange. However, it has a long history. The ancient Greek doctor Hippocrates – the father of medicine – is credited with saying that "All disease begins in the gut". Similarly, the 4th-century Chinese doctor Ge Hong described the use of "Yellow Soup", a euphemism describing ingestion of dried fecal material to treat severe diarrhea. I was introduced to this research field as a result of my work at the Centre for Clinical Research. Originally, the department worked with the role of infectious organisms in cancer, especially the human papillomavirus. With this background, I began to conduct a study investigating whether certain known infectious bacteria could lead to colorectal cancer. While I observed the bacteria in cancer tissue samples, what I found more interesting was a marked reduction in these bacteria in precursor lesions when compared to both healthy and cancerous tissue. Could it be that the absence of bacteria could lead to changes in the tissue? This put me on a path towards the research field of gut microbiota. Hopefully, after you have read this thesis, you will feel more informed about this subject and will understand my fascination with the highly complex world of the interaction between body and microbiota.

The last four years have been a journey, with many unique challenges. Along the way, I have had to learn bioinformatics algorithms, which has allowed me to be involved in several other fascinating projects at my department. I have talked with many families for whom ADHD and ASD were a large part of life. Overall, I can confidently say that I have matured and become a more independent researcher. While a Ph.D. project is hard work, I am confident that I will remember it fondly.

The project described in this thesis was carried out at the Centre for Clinical Research, North Denmark Regional Hospital/Aalborg University, Denmark in collaboration with the Department for Child and Adolescent Psychiatry, as well as the Research Unit for Child and Adolescent Psychiatry, both at Aalborg University Hospital, Denmark. Funding was kindly provided by the North Denmark Regional Hospital, Marie Jensen og Jensine Heibergs Fond, Region Nordjylland Sundshedsvidenskabelige Forskningsfond, Grosserer L. F. Foghts Fond, EliteForsk Travel Grant, Fru C. Hermansens Mindelegat, Niels Jensens Forskningslegat, and Sofiefonden. This study would not have been possible without this assistance.

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A Ph.D. thesis like this is rarely the work of a single person. As such, I have been lucky to have the support of colleagues, family, and friends.

First and foremost, I would like to say a special thank you to my supervisor team and my primary supervisor, Suzette Sørensen, for all her guidance and the great scientific discussions. I would like to thank Søren Hagstrøm - all laboratory personnel should have a clinician like you to assist with how to handle recruitment, patients, and the clinical world. Also, I would like to thank Mette Nyegaard for technical discussion and assistance with critically evaluating the results. Your help and advice have been highly appreciated. I will always fondly remember meetings scheduled for two hours but ending up lasting several hours longer because we just kept coming up with fun new ideas. Hopefully, there can be room for collaborations in the future!

I would also like to thank the current and previous staff at the Centre for Clinical Research, North Denmark Regional Hospital for academic discussions when it came to their technical assistance and making my time in the center highly enjoyable. A special thanks go out to our excellent technical staff: Ann-Maria Jensen, Bente Jensen, Signe Østergaard, and Anne Sofie Vedsted for all their help with receiving and handling samples, assistance with laboratory procedures, as well as technical discussions.

Finding study participants is always a challenge in biomedical studies. Therefore, I would like to thank the Department for Child and Adolescent Psychiatry, Aalborg University Hospital for their great assistance with the recruitment of families for the project. As part of my recruitment efforts, I was warmly welcomed at the Research Unit for Child and Adolescent Psychiatry, Aalborg University Hospital. Thank you for accepting a laboratory scientist into a clinical research group, and for always helping me keep a focus on the clinical side of the research. Special gratitude goes to Marlene Lauritsen for all the advice provided, as well as to Heidi Hattmann for assistance with obtaining the patient records.

A special thanks go out to all the families that have participated in this project. It could not have been completed without them. Even though they received no personal gains from participating, the families have shown a great interest in this project and gave me a great deal of insight into their personal lives.

Finally, I would like to express my gratitude to friends and family. You have listened to my stories, my frustrations, as well as my achievements – even when they might not have made any sense for you. You have been patient when I have been busy and have always been available when I needed distraction. I would not have been able to complete this project without your support.

ENGLISH SUMMARY

Background: Attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) are neurodevelopmental disorders with a high degree of clinical overlap. Despite both disorders having a clear hereditary background, genetics alone has been unable to accurately predict all cases of ADHD and ASD. Instead, studies suggest that genetic-environmental interactions are behind these disorders. For both disorders, gastrointestinal and immunological problems are common, which points to a role played by the intestines in ADHD and ASD. Given the growing evidence of the role of gut microbiota in neurodevelopment, as well as in other neurological and psychiatric disorders, studies are beginning to investigate the role of the gut microbiota in ADHD and ASD.

Aim: Based on the above, the overall aim of this Ph.D. thesis was to investigate the involvement of the gut microbiota in ADHD and ASD. For this purpose, an initial study was conducted aiming to evaluate storage methodologies suitable for home-sampling feces for gut microbiota evaluation. Secondly, a systematic review was performed, aiming to provide an overview of studies investigating the gut microbiota in ADHD and ASD. These two studies lead to a third study that aimed to examine gut microbiota in parallel in children with ADHD and/or ASD, as well as to evaluate pathways for how gut microbiota influences the body.

Materials and methods: The initial methodological study utilized fecal samples from three donors. From each donor feces, 45 fecal replicas were taken and stored either frozen at -80 °C or -20 °C, or in one of three separate storage buffers at 4 °C or room temperature. All storage conditions were maintained for 24 or 72 hours, after which bacterial composition was investigated using amplicon sequencing of the V4 region on the 16S rRNA gene. The systematic literature review was based on a systematic search on PubMed and Embase. This search identified four studies investigating gut microbiota in ADHD, and 20 studies for ASD. The final study collected fecal and blood plasma samples from children with either ADHD (n=32), ASD (n=12), or comorbid ADHD/ASD (n=11) or as controls, non-affected siblings (n= 14, 5, and 11 for siblings of children with ADHD, ASD, or comorbid ADHD/ASD, respectively), and non-related children (n=17). For all study participants, gut microbiota was assessed using amplicon sequencing of the V4 region on the 16S rRNA gene. For indicators of gastrointestinal permeability, lipopolysaccharide-binding protein (LBP) was investigated in the blood plasma, while calprotectin was investigated in fecal samples.

Results: Results from the initial methodology study revealed that while small variations might occur, both freezing, as well as the use of storage buffers could maintain gut microbiota signatures of the individual donors. Overall, freezing

samples at -20 °C most closely resembled that of fecal samples stored at -80 °C. Therefore, this was chosen as the storage methodology for further studies. The systematic literature review revealed an overall agreement between studies on an association between gut microbiota and ASD although a specific gut microbiota signature could not be identified. For ADHD, results were highly heterogeneous, and thus no clear association between ADHD and gut microbiota variations could be established. For both disorders, highly heterogenous results hampered the ability of this study to compare individual study results. Notably, despite the clinical overlap, no studies were identified that investigated and compared the gut microbiota in individuals with ADHD and ASD in parallel. Finally, in the clinical study, the gut microbiota of individuals with ADHD and/ASD were observed to share a gut microbiota signature, distinct from that of non-related controls. Furthermore, both ADHD and/or ASD showed indicators of increased gastrointestinal permeability as indicated by increased LBP concentrations in certain cases.

Conclusions: The results of this project confirm that children with ADHD and/or ASD possess a gut microbiota distinct from that of non-affected children. A novel finding of this project is that gut microbiota variations are shared between individuals with ADHD and ASD, indicating that processes common to both disorders are associated with gut microbiota variations either as a cause or an effect. Finally, in an initial step for investigating how these bacteria influenced the body, we showed that children with ADHD and/or ASD had indicators of increased gastrointestinal barrier permeability. More studies are needed to uncover how this interacts with the presentations and comorbidities in ADHD and ASD.

DANSK RESUME

Baggrund: Attention-deficit Hyperactivity Disorder (ADHD) og autismespektrums-forstyrrelser (autisme) er udviklingsforstyrrelser med en høj grad af klinisk overlap. Begge tilstande er nedarvede men på trods af dette, kan genetik alene ikke forklarer alle tilfælde. En række studier har derimod indikeret at ADHD og autisme opstår som resultat af en interaktion mellem arv og miljø. Eftersom mavetarmproblemer og immunrelaterede sygdomme er udbredte for begge diagnoser, har der samlet sig en interesse for om elementer i tarmsystemet kunne være indblandet i ADHD og autisme. Flere og flere studier har det seneste årti fundet beviser for, at tarmens bakteriesammensætning (tarmmikrobiotaen) har en vigtig rolle i udvikling af nervesystemet og hjernen, mens forstyrrelser af tarmmikrobiotaen er blevet påvist i flere neurologiske og psykiatriske sygdomme. Med denne baggrund, er der kommet en stigende interesse for hvorvidt tarmmikrobiotaen kunne spille en rolle i udviklingsforstyrrelserne ADHD og autisme.

Formål: Formålet med denne Ph.d.-afhandling er at undersøge hvorvidt ADHD og autisme er forbundet med en anderledes tarmmikrobiota. Et initialt metodestudie udføres for at evaluerer metoder der kan tillade opbevaring af afføringsprøver hjemme ved forsøgspersoner. Herudover udføres en systematisk litteraturgennemgang for at afdække den eksisterende viden om tarmmikrobiotaen i ADHD og autisme. Baseret på erfaringerne fra disse to studier, udføres et tredje observationelt studie. Formålet med dette er at undersøge og sammenligne tarmmikrobiotaen i børn med ADHD og/eller autisme.

Materialer og metoder: Metodestudiet var baseret på afføringsprøver fra tre donorer. Fra hver donorafføring blev der udtaget 45 prøver, som herefter blev opbevaret på en af følgende måder: Frossent ved -80 °C (guldstandarden), frossent ved -20 °C, eller i en af tre opbevaringsbuffere ved enten 4 °C eller stuetemperatur. Alle opbevaringsmetoder blev opretholdt for 24 og 72 timer, hvorefter mikrobiotaen blev afdækket med amplikon sekventering rettet mod V4 regionen på 16S rRNA genet. Den systematiske litteraturgennemgang var baseret på en systematisk søgning på PubMed og Embase udført 22. juli 2019. Her blev fire studier omkring tarmmikrobiotaen i ADHD identificeret, og 20 studier omkring tarmmikrobiotaen i autisme. Disse studier blev sammenlignet ud fra demografiske data, metoder, samt variation i mikrobiota. I det observationelle studie III, indsamledes blodplasma og afføringsprøver fra børn med enten ADHD (n=32), autisme (n=12), samtidig ADHD og autisme (n=11), søskende uden diagnoserne (n= 14, 5, and 11 for søskende til børn med ADHD, autisme eller samtidig ADHD og autisme) eller ikke-relaterede børn uden diagnoserne (n=17). Tarmmikrobiotaen blev undersøgt ved hjælp af amplikon sekventering af V4 regionen på 16S rRNA genet, mens tarmvæggens integritet blev målt med ELISA rettet mod LBP i plasma og calprotectin i afføring.

Resultater: Det metodologiske studie viste at både frysning samt brug af buffere kunne opretholde den donorspecifikke bakteriesignatur, trods mindre variationer indenfor samme donor. Frysning af afføring ved -20 °C var mest effektiv til at reproducerer bakteriesammensætningen af afføring opbevaret ved -80 °C, og blev derfor valgt som opbevaringsmetode til tarmmikrobiotastudier. Den systematiske litteraturgennemgang viste at tidligere studier generelt indikerer at autisme er forbundet med en anderledes tarmmikrobiotasammensætning i forhold til kontroller, men en høj grad af variabilitet mellem studierne, umuliggjorde identifikationen af en autisme-specifik bakteriesignatur. Kun fire studier havde undersøgt tarmmikrobiotaen i ADHD på daværende tidspunkt, og der var ikke enighed mellem dem omkring resultaterne. Trods tidligere indikationer for klinisk overlap mellem diagnoserne, blev der ikke fundet studier der undersøgte tarmmikrobiotaen parallelt i ADHD og autisme. Dette blev undersøgt i studie III, hvor vi observerede at tarmmikrobiotaen i børn med ADHD og/eller autisme, delte en tarmmikrobiota signatur der var forskellig fra kontroller. Herudover observerede vi, at børn med ADHD og/eller autisme havde signifikant øget LBP i plasma, hvilket indikerer en gennemtrængelighed af tarmvæggen.

Konklusioner: Resultaterne fra dette projekt bekræfter at børn med ADHD og/eller autisme har en tarmmikrobiota signatur der er forskellig fra børn uden disse diagnoser. Et nyt fund i dette studie er, at denne signatur var delt for ADHD og autisme. Dette indikerer at processerne bag tarmmikrobiota variationen er til stede i begge diagnoser, enten som årsag eller virkning. Som et første trin i afdækningen af hvordan tarmmikrobiotaen potentielt kan påvirke kroppen i børn med ADHD og autisme, viste vi at produkter fra tarmbakterierne kan passere over tarmvæggen i børn med disse diagnoser. Betydningen af dette for symptomer og komorbiditeter i personer med ADHD og autisme, er endnu ukendt.

TABLE OF CONTENTS

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following three papers:

Paper I: C. Bundgaard-Nielsen, S. Hagstrøm, S. Sørensen. Interpersonal Variations in Gut Microbiota Profiles Supersedes the Effects of Differing Fecal Storage Conditions. *Scientific Reports* 8(1). 2018. doi:10.5061/dryad.61r43kd.
(This paper is published)

Paper II: C. Bundgaard-Nielsen, J. K. Knudsen, P. D. C. Leutscher, M. B. Lauritsen, M. Nyegaard, S. Hagstrøm, S. Sørensen. Gut microbiota profiles of autism spectrum disorder and attention deficit/hyperactivity disorder: A systematic literature review. *Gut Microbes* 11(5). 2020. doi:10.1080/19490976.2020.1748258
(This paper is published)

Paper III: C. Bundgaard-Nielsen, M. B. Lauritsen, J. K. Knudsen, L. S. Rold, P. Hindersson, P. D. C. Leutscher, S. Hagstrøm, M. Nyegaard, S. Sørensen. Children with Attention-Deficit Hyperactivity Disorder or Autism Spectrum Disorder Share Distinct Microbiota Compositions and gastrointestinal permeability.
(This paper is in preparation, and has been made available for the Ph.D. committee)

TABLES AND FIGURES

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ABBREVIATIONS

ADHD	Attention-Deficit Hyperactivity Disorder
ADHD-RS	ADHD Rating Scale
ADOS2	Autism Diagnostic Observation Schedule 2nd edition
ASD	Autism Spectrum Disorder
ASV	Amplicon Sequence Variants
DNA	Deoxyribonucleic Acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELISA	Enzyme-Linked Immunosorbent Assay
FEIA	Fluorescent Enzyme Immunoassay
GABA	Gamma-Aminobutyric Acid
HPA	Hypothalamus-Pituitary-Adrenal
HRP	Horse Radish Peroxidase
ICD	International Statistical Classification of Diseases and Related Health Problems
LBP	Lipopolysaccharide-Binding Protein
LPS	Lipopolysaccharide
LEfSe	Linear Discriminant Analysis Effect Size
MRI	Magnetic Resonance Imaging
NOS	Newcastle-Ottawa Scale
PCA	Principal Component Analysis
PCoA	Principal-Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SCFA	Short Chain Fatty Acids
T.O.V.A.	Test of Variables of Attention

CHAPTER 1. BACKGROUND

Attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) describe a range of neurodevelopmental disorders that are common in children and adolescents[1]. Recently, studies have started to reveal the importance of components of the gastrointestinal system in these disorders[2,3]. In the following section, first ADHD and ASD are described, followed by a description of the microbiota involved and how these may impact neurodevelopmental disorders. This will form the theoretical basis for the Ph.D. thesis investigating gut microbiota in individuals with ADHD and ASD.

1.1 ADHD AND ASD

1.1.1 PREVALENCE AND SYMPTOMS

ADHD is a neurodevelopmental disorder, characterized by varying degrees of inattention, impulsivity, and hyperactivity[4,5]. Inattention is defined as difficulties with maintaining attention for tasks that do not provide frequent stimuli or rewards. Impulsivity refers to a tendency to act on stimuli without considering risks. Finally, hyperactivity is related to increased physical activity, typically in situations where this is not suitable (e.g., in school)[4]. Based on the dominating symptoms, the Diagnostic and Statistical Manual of Mental Disorders (DSM)-5[5], as well as the new International Statistical Classification of Diseases and Related Health Problems (ICD)-11 (expected to be implemented in 2022)[4], divide ADHD into three different presentations: 1) inattentive, hyperactive, 2) impulsive 3) a combination of the two. In comparison, the current ICD-10 differentiates between attention deficit with and without hyperactivity[6]. The presentation of symptoms may vary over time[4,5], with some children becoming symptom-free later in life[7,8]. Typically, symptoms of ADHD manifest before the age of 12[4,5] although there are variations depending on the ADHD type and the child's gender. For ADHD types involving hyperactivity, incidence peaks at the age of 8 years for males, whereas the inattentive ADHD presentation peaks around the age of 11 years for males. ADHD is typically diagnosed later in girls compared to boys, with the incidence of both inattentive- and hyperactive-dominated ADHD peaking at the age of 15 years for females[9]. ADHD is one of the most common disorders within childhood and adolescent psychiatric departments, with a global prevalence of 7.2 % of children meeting the DSM-IV criteria[10]. However, the prevalence varies across different geographical regions, age groups, and genders, with childhood-onset ADHD being more commonly diagnosed in males compared to females[8,10,11]. It is unclear whether these variations are true differences or represent differences in clinical presentation or diagnostic criteria. [11,12].

The diagnosis of ASD describes a spectrum of neurodevelopmental disorders. The exact presentation of the disorder varies between individuals. Overall, ASD is characterized by varying degrees of deficiencies in social and emotional skills, as well as difficulties with communication combined with a tendency for irritability and restrictive-repetitive behaviors[4,5]. Several types of autism have been described in the currently used ICD-10, including Asperger's syndrome, childhood autism, or atypical autism[6]. These have been combined to form the ASD diagnosis in the DSM-5 and the new ICD-11[4,5]. Symptoms have often been present from an early age and most receive their diagnosis soon after starting school[13]. However, the age of diagnosis is highly dependent on the severity of symptoms and gender, with a Danish study showing that for males, the incidence rates peaked around the age of 5 years and stayed constant until the age of 14, whereas for females, the incidence peaked around the age of 14 years[9]. The prevalence of ASD among children is estimated to range from 0.3-1.2 %, although as for ADHD, the exact prevalence varies between different geographical regions, age groups, and gender, with ASD being more common among males[14].

While the two disorders have some overall differences, several similarities have also been observed. Both disorders are neurodevelopmental disorders, typically diagnosed in early childhood[4,5,9,13], and are more often diagnosed in males than females[11,14]. Until recently, the diagnosis of ASD precluded a subsequent diagnosis of ADHD[15]. However, symptoms associated with ADHD were commonly reported amongst children with ASD[16,17], and thus in the DSM-5[5] as well as the ICD-10/11[4,6], comorbid ADHD and ASD were allowed. Subsequently, a large study reported that up to 62.7 % of children with ASD also presented clinical symptoms of ADHD[18], whereas Sokolova *et al*.[19] suggested that symptoms of ADHD and ASD were interconnected in children with comorbid ADHD and ASD[19]. To investigate the association between the two disorders, a twin study by Ronald *et al*.[20] demonstrated a high degree of shared genetic influences between ADHD and ASD. This has subsequently been substantiated by other studies reporting a similar burden of genetic variants in the two disorders[21]. Overall, despite differences in symptomology, the diagnoses of ADHD and ASD have a large clinical and hereditary overlap.

1.1.2 TREATMENT OF ADHD AND ASD

Untreated, ADHD is associated with a significantly higher risk of emotional and psychiatric disorders[22], as well as reduced work performance, increased financial stress[23], and an increased tendency for drug abuse and dependency during adolescence and adult life[22,23]. Treatment typically consists of a combination of pharmacological and psychosocial interventions[24], with the most common type of pharmacologic treatment consisting of stimulants like methylphenidate or dexamphetamines[25,26]. These function by increasing dopamine and noradrenalin-based signaling in the prefrontal cortex[27]. Although effective in the treatment of many cases of ADHD[26,28],

the benefits are not universal[26]. Together with worries concerning side effects and problems with adherence, this has led to attempts to utilize non-pharmacological intervention strategies as complementary treatment options in ADHD. Famously, dietary interventions have been utilized with some success in ADHD[29] although overall outcomes of intervention studies have proven inconsistent[30]. As such, to develop better intervention strategies, more knowledge is needed on the causes of ADHD.

Symptoms of childhood ASD tend to remain stable into adulthood[31], with around 47.7 % requiring significant support for maintaining everyday life[32]. As a result, emotional disorders and problems with education and employment are common for adults with ASD[33,34]. An effective treatment is thus urgently needed. Current treatment approaches are focused on psychosocial therapy, including behavioral therapy, although the effectiveness, unfortunately, varies between patients. Pharmacological treatment is sometimes included to treat emotional symptoms in the affected children although no pharmacological interventions exists for the treatment of the core symptoms of ASD[35]. Some attempts have been made to implement casein-free and gluten-free diets in the treatment of ASD although with varying success[36].

1.1.3 PATHOPHYSIOLOGY

Neural variations

Structural and functional imaging technology has provided a better representation of the neurodevelopmental processes in children with ADHD and ASD. Three distinct ADHD subgroups have been described by Stevens *et al*.[37] based on diagnostic tests, as well as functional magnetic resonance imaging (MRI). These consisted of children with deficiencies in reward anticipation, children with deficiencies in impulsivity, and children with normal results in these tests. Notably, brain abnormalities were distinct for each group, with no shared ADHD-specific variation observed. Thus, care should be taken when trying to extrapolate neuroimaging findings to all children with ADHD. Other researchers have similarly reported deficiencies in reward anticipation pathways in children with ADHD[38]. This deficiency in reward anticipation has been linked to a reduction in synaptic dopamine[39], which is in agreement with the positive effects of stimulant medication increasing the intrasynaptic dopamine[27].

As a neurodevelopmental disorder, ASD is characterized by atypical brain development. During the first few years following birth, MRI has revealed that the brains of children with ASD undergo an initial abnormally fast growth, which is then followed by a period of stagnation[40]. Due to this early disruption of brain development, several different regions are affected, leading to an overall difference in cortical thickness and surface area across the brain[41]. These structural differences may

result in the differences in brain activity as reported for ASD in response to facial processing[42], as well inefficient neural connectivity, leading to a tendency for hyper-arousal during tasks requiring attention[43]. These structural observations are, however, not observed universally in individuals with ASD, which is in agreement with its status as a spectrum of disorders. Furthermore, increased blood and brain concentrations of glutamate have been observed in children with ASD[44]. While this neurotransmitter is normally important for cognitive functioning, excess glutamate has been demonstrated to induce neural cell apoptosis[45].

Genetics

Sibling and twin studies have revealed that both ADHD and ASD are highly hereditary disorders[46,47], with some overlap in genetic influence between the two[21,48]. For ADHD, studies have reported variations in genes involved in the neurotransmitter pathways involving serotonin[49–51] and dopamine[51–53], which is consistent with the observed disturbances on dopamine signaling seen in ADHD. A recent genome-wide association study identified 12 genome-wide significant loci, most of which were in genes related to brain development. However, these genes could only predict around 22 % of heritability[54] compared to 74 % heritability estimated from twin studies[55]. Therefore, it is clear that heritability in ADHD is highly polygenic. Furthermore, heterogeneity has been seen amongst observed risk genes[56]. Overall, while ADHD is a disorder with clear genetic involvement, genetics alone cannot sufficiently explain all cases of ADHD, and instead, interactions between genetic and environmental factors are suspected[51].

Similar to ADHD, the genetic background for ASD has been shown to depend on the combination of several genes, often common variants[57], that together predispose for different symptoms of ASD[58–60]. Several of the identified gene variants are normally involved in neural transmission and development[59,60]. Despite the clear genetic involvement, genetics alone does not sufficiently explain all cases of ASD, with twin studies showing that environmental factors might account for up to 55 % of variations when it comes to the risk of the disorder[47].

While genetic factors have been shown to explain most cases of ADHD and ASD, both have clear environmental involvements. For both ADHD and ASD, low birthweight is a common risk factor[61,62] suggesting an effect during the fetal state. Furthermore, dietary components have been shown to cause a worsening of symptoms in cases of ADHD[63]. However, no environmental effect has been proven causal, and it is thus clear that we still lack information on the role of environmental factors in ADHD and ASD. Based on these observations, as well as the experiences with dietary interventions, studies have started to examine whether components of the

gastrointestinal tract could be involved in the pathophysiology of neurodevelopmental disorders[64].

1.2 GUT MICROBIOTA

1.2.1 NORMAL GUT MICROBIOTA

The human body is home to numerous microorganisms, which includes bacteria but also viruses, fungi, and archaea. These are collectively known as the microbiota, and their collective genomes are referred to as the microbiome[65,66]. It has been estimated that a normal human male contains a total of $3.8x10^{13}$ bacteria comparable to the $3.0x10^{13}$ estimated human cells ($0.3x10^{13}$ without erythrocytes). The majority of these bacteria is located in the large intestines where, together with other microorganisms, they are referred to as the human gut microbiota[67]. Several functions and disorders have been associated with the gut microbiota, and thus this area has been heavily investigated over the last decade[66]. Due to the ease of acquisition, most studies describe gut microbiota using the bacterial composition of fecal samples. These are, however, surrogate markers, as previous studies have reported variations between the fecal microbiota and intestinal mucosal microbiota in the same individuals[68,69]. Several large-scale studies have attempted to describe the composition and variation of normal gut microbiota, including the MetaHit Consortium[70], Human Microbiome Project[71], as well as the newer American Gut[72]. For normal healthy adults, the gut microbiota is dominated by bacteria belonging to the phyla Firmicutes and Bacteroidetes, with contributions from Actinobacteria and Proteobacteria[65,70,73–76]. At the genus level, *Bacteroides, Alistipes, Eubacterium, Prevotella*, and *Faecalibacterium* dominate in healthy adults[65,73,77] (see figure 1). Besides these dominant bacterial taxa, several less abundant taxa have also been identified with individual variations. Arumugam *et al.*[78] demonstrated that the gut microbiota of healthy individuals could be roughly divided into three distinct enterotypes based on variations in the relative abundance of the genera *Bacteroides*, *Prevotella*, and *Ruminococcus*[78]. While these identifying bacteria are not necessarily the most common genera within each enterotype, each enterotype instead describes specific bacterial clusters that differ from each other depending on the route employed for generating energy[78]. Improved sequencing methodologies have, however, revealed that there are still gaps in our knowledge concerning the composition of gut microbiota. As demonstrated by Almeida *et al.*[74], 74 % of bacterial families and 40 % of bacterial genera observed during sequencing are yet to be identified. Indeed, as sequencing technology improves and full genome sequencing techniques become

more widely implemented, it is expected that more bacterial genera and species will be identified, resulting in a higher resolution[74].

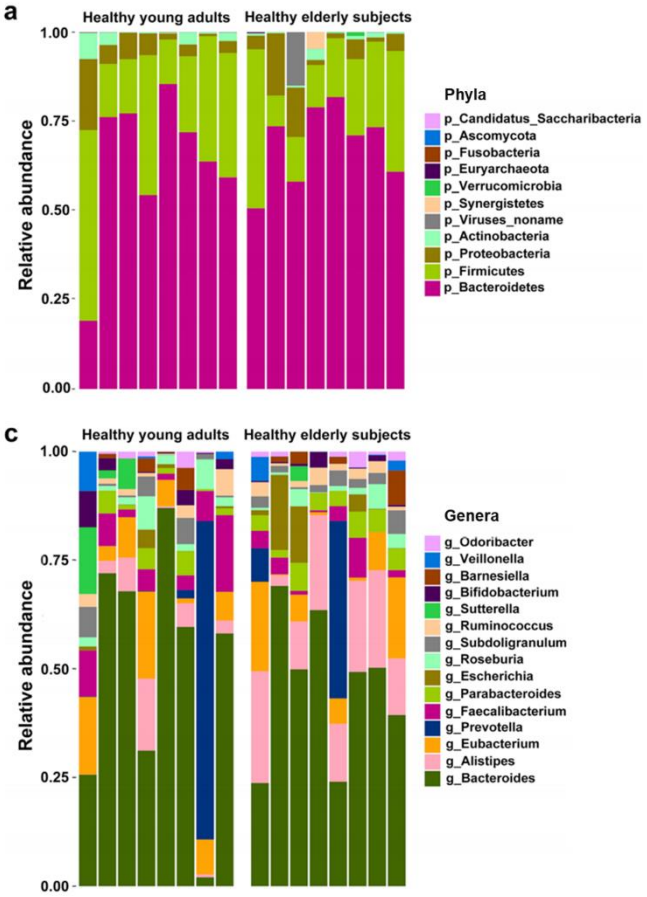

Figure 1: Gut microbiota composition at phylum and genus taxonomic levels in eight healthy young adult males and females (mean age 33.3 years) and eight healthy elderly males and females (mean age 71.6 years). The high degree of individual variation in gut microbiota is evident here. Adapted from Li et al.[73].

1.2.2 DEVELOPMENT AND NORMAL VARIATION WITHIN THE GUT MICROBIOTA

As indicated in figure 1, small variations of gut microbiota can be identified depending on age[73]. Gut microbiota is believed to be established through maternal vertical transmission[79] although the time of the initial colonization is disputed. Based on the observation of bacteria in amniotic fluid[80] and the placenta[81], *in utero* colonization has been suggested, although the validity of these findings have been questioned[82,83]. Despite this dispute, it is commonly accepted that birth is a major event in establishing the infant gut microbiota. Immediately following birth, the major sources of microbiota in vaginally delivered infants are the maternal gut and vaginal

microbiota[79,84]. In comparison, the gut microbiota of infants born by cesarean section is predominately cultured by bacteria from the maternal skin[84]. Gradually, facultative anaerobic bacteria originating from the maternal gut microbiota start to dominate the infant gut microbiota[79], and gradually, the gut microbiota of children delivered vaginally and through cesarean section, start to bear the same resemblance[85]. During the next weeks, gut microbiota in breast-fed children starts to become dominated by bacteria capable of digesting oligosaccharides from human breastmilk like *Bifidobacterium, Lactobacillus,* and *Clostridium*[85,86], whereas gut microbiota in formula-fed children obtains a higher relative abundance of *Bacteroides*[87] and *Clostridium* species[86,87]. The gradual introduction of solid food causes another major shift in gut microbiota, with a reduction in the relative abundance of *Bifidobacterium,* while *Bacteroides* becomes a dominant bacteria[86]. During the following years, the child's gut microbiota continues to develop until the age of three where it resembles the adult gut microbiota. Hereafter, the gut microbiota remains stable during later childhood, adolescence, and adulthood in the absence of external influences[88].

While gut microbiota is considered fairly stable once established[88], several lifestyles or health factors are capable of changing it. Long-term dietary variations, in particular, has been considered a major influencer of gut microbiota[89–93]. David *et al*.[92] were able to link specific diets with distinct gut microbiotas, as well as demonstrating that diet was effective in changing gut microbiota by modifying the available nutrients. Indeed a fiber-rich, plant-dominated diet has been associated with gut microbiota rich in phyla Bacteroidetes and Actinobacteria[90,91], as well as genera *Actinobacteria*[90,91], *Prevotella*[90–93], and *Xylanibacter*[90,93]. These bacteria are effective in fermenting dietary fibers[90]. Conversely, a diet rich in saturated fat / low in dietary fibers has been associated with a gut microbiota dominated by the phyla Firmicutes and Proteobacteria[90,91], as well as the genus *Bacteroides*[91]. In mice, Sonnenburg *et al*.[94] observed that, although changes to gut microbiota following dietary modifications could be reversed, the long-term consumption of low-fiber diets could lead to lasting modifications in gut microbiota. Besides differences in the bacterial capacity to digest fibers, a fiber-rich diet may also influence gut microbiota through the intestinal passage time. During defecation, a large portion of the resident bacteria in the colon is washed out. Therefore, to maintain their contribution to the gut microbiota, bacterial growth needs to match the loss during defecation. As a result, a rapid intestinal transit time is associated with gut microbiota being dominated by rapidly growing bacteria[95].

Besides diet, other differences in lifestyle have also been shown to influence gut microbiota. In a study conducted in a recently urbanized Chinese population, Winglee *et al*.[89] observed that urbanization led to reduced bacterial diversity and increased the relative abundance of *Escherichia* and *Shigella* compared to the nearby rural population, even after controlling for differences in diet. Other factors like the use of drugs or other external chemical factors can have a large influence on gut microbiota. This is especially true for antibiotics, which can have profound and long-lasting effects on gut microbiota. These do not stabilize until after two months[96]. Also, less

obvious drugs can influence gut microbiota, with one large study demonstrating that around 24 % of the >1000 drugs tested could influence bacterial growth in gut microbiota. A disproportionate number of these were antipsychotic drugs, but antidepressants, hormones, and immunosuppressants were also represented[97]. Overall, several intrinsic and extrinsic factors can influence gut microbiota, and thus, care must be taken to eliminate confounders when investigating gut microbiota.

1.2.3 GUT MICROBIOTA FUNCTIONS

The gut microbiota is a living organism that constantly reacts and adapts to its environment. During human evolution, the gut microbiota has developed with us, and thus, we have adapted to coexisting[98]. As a result, the gut microbiota has been implicated in several normal functions as indicated in figure 2. The most obvious function of gut microbiota is its role in digesting food compounds. Bacteria in normal gut microbiota are capable of fermenting dietary fibers[90,99], and in the process, producing short-chain fatty acids (SCFAs) like butyrate. Butyrate, in turn, serves as the main energy source for enterocytes[100], while several other SCFAs have beneficial effects for the human body, including maintaining glucose control[99]. Besides SCFAs, the gut microbiota has important roles in the biosynthesis of essential amino acids[101] and vitamins[102].

In addition to assisting with digestion, healthy gut microbiota can provide resistance to pathogenic bacteria. By competing for nutrients, healthy gut microbiota prevents colonization or overgrowth of pathogens[103,104]. Other bacteria are capable of fighting pathogenic bacteria through the conversion of bile acids into the antimicrobial metabolite, deoxycholic acid[105,106], or the inactivation of virulence factors[107].

While the diet and lifestyle of the host can influence the development of gut microbiota, the gut microbiota also has a unique ability to ensure correct development of the human intestinal epithelium, as well as the maturation of the immune system. During early life, developing gut microbiota stimulates the production of a mucin layer, separating the epithelial cells from the intestinal lumen[108]. In later life, healthy gut microbiota maintains this epithelium by protecting against excess inflammation[109], and by increasing the production of tight junction proteins, thus maintaining an effective intestinal barrier[110]. While the intestinal epithelial barrier effectively prevents the entrance of gut bacteria, gut microbiota interacts closely with the intestinal immune system through the presence of toll-like receptors that are important in regulating the immune response[111], as discussed in a review by Zheng *et al*[112]. Briefly, experiments on germ-free mice have shown that mice lacking gut microbiota have several immune deficiencies, including a low lymphocyte count. Importantly, these deficiencies can be partly normalized following transplantation with normal gut bacteria[113]. Further studies have revealed, that gut microbiota

stimulates the maturation of regulatory B and T cells, thereby regulating inflammation[114,115].

Overall, the gut microbiota has several important effects, and thus, maintaining healthy gut microbiota is essential for normal homeostasis.

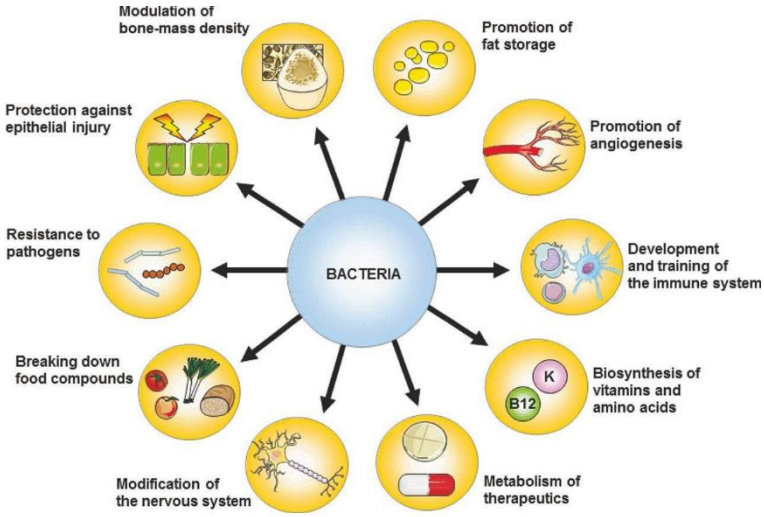

Figure 2: Overview of biological functions attributed to gut microbiota. Reprinted with permission from Laukens et al.[116]

1.2.4 GUT-BRAIN AXIS

Under normal conditions, the gut microbiota has a bidirectional interaction with the brain as summarized in figure 3.

The Vagus nerve is a major cranial nerve that distributes sensory signals from the gastrointestinal tract to the central nervous system, as well as efferent signals regulating the peripheral functions[117]. Two studies have reported that oral supplementation of the probiotic bacteria *Lactobacillus rhamnsus* led to increased expression and production of the neurotransmitter gamma-Aminobutyric acid (GABA) in the brains of mice. This was directly associated with a reduction in anxiety and depression-like behavior[117,118]. This signaling was counteracted by vagotomy, indicating that *L. rhamnsus* interacted with the brain expression of GABA through the Vagus nerve[117]. Similarly, afferent nerves can be directly activated by the microbiota-produced SCFA butyrate[119]. Several neurotransmitters are produced in the gut, with certain bacteria capable of synthesizing dopamine[120–122] and noradrenalin[120], while 90

% of serotonin is produced in the gastrointestinal tract by colonic enterochromaffin cells in a process stimulated by gut microbiota metabolites[123,124]. It is not fully understood whether disruptions in intestinal production of these neurotransmitters translate to altered concentrations in the brain, but the turnover of dopamine and serotonin has been demonstrated to be higher in the brains of germ-free mice[125].

Besides neurotransmitters, endocrine[126] and immunological signals[127] have also been demonstrated in the gut-brain axis. The most important endocrine pathway is through the Hypothalamus-Pituitary-adrenal (HPA) axis that regulates the stress response. Here, Sudo *et al*. [126] demonstrated that germ-free mice had elevated HPA activity, which could be reversed by inoculation with *Bifidobacterium infantis*.

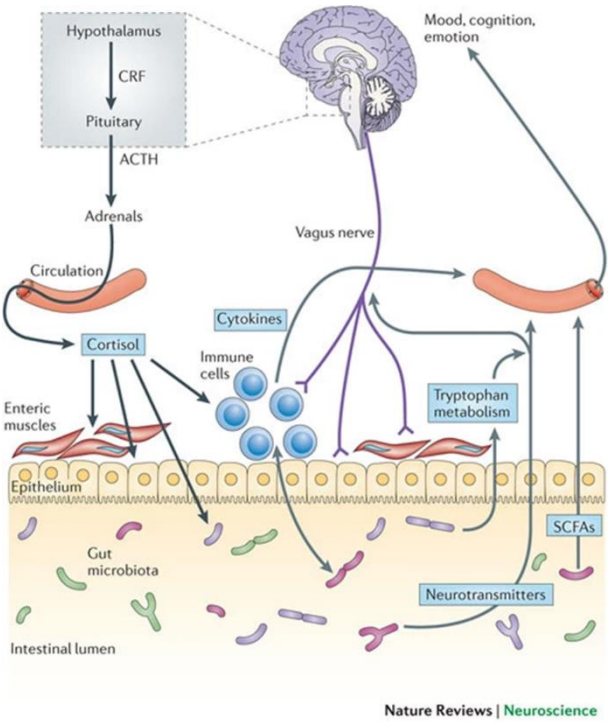

Figure 3: Overview of the individual mediators of the gut-brain axis that describes the bidirectional interaction between gut microbiota and the brain. These include signaling through the Vagus nerve, the production of psychoactive metabolites, and immune stimulation. Reprinted with permission from Cryan et al[128].

Experiments on germ-free mice have shown that in the absence of gut microbiota, normal brain development was hampered[129,130] whereas transplantation of gut microbiota could increase markers of neural development[131]. This was supported by a larger cohort study that reported that children with gut microbiota dominated by

members of the Clostridiales order possessed poorer social, personal, and communicative skills[132]. Children who received antibiotic treatment during the first 24 months of life have been shown to have worse neurocognitive outcomes at the age of 11[133]. While it was not clear whether this was due to the effects of antibiotic treatment or whether antibiotics may be a confounder, it demonstrates that gut microbiota may have a role in neurodevelopment in both mice and humans.

Importantly, most of the available knowledge concerning the gut-brain axis originates from animal studies, and thus, it is still unclear to what degree this relates to humans.

1.2.5 DYSBIOSIS, LEAKY GUT, AND THE INVOLVEMENT OF GUT MICROBIOTA IN DISEASE

Since the gut microbiota is involved in maintaining several important normal functions, disruptions can impact these functions or lead to overgrowth of pathogenic bacteria, ultimately leading to disease. The presence of an unbalanced or disease-promoting gut microbiota has been referred to as dysbiosis, although the definition is broad and ill-defined[134]. Dysbiosis has been implicated in numerous diseases and conditions, including overgrowth of pathogenic bacteria[135], inflammatory bowel disorders[136], multiple sclerosis[137], obesity[138,139], as well as psychiatric conditions like major depressive disorder[140,141] or bipolar disorder[142].

The direction of interaction between dysbiosis and the associated disease differs. Some conditions can influence the gut microbiota by actively changing its growth conditions. This includes diarrhea, where the increased passage time favors rapidly reproducing bacteria[95,143], as well as intestinal inflammation that promotes loss of several bacteria[144]. The use of orally administered drugs, in the treatment of some diseases, can affect the gut microbiota. This is especially true for antibiotics, which can drastically alter the gut microbiota[96,135], but also non-antibiotic drugs can inhibit the growth of common gut bacteria, as demonstrated by Maier *et al*[97]. Conversely, in other disorders, dysbiosis may directly lead to disease. This is especially true for infectious diseases. Disruption of the normal gut microbiota can leave the gastrointestinal system susceptible to infection and overgrowth with pathogenic bacteria, which is normally kept in check by the resident bacteria[96,103,104]. These can subsequently lead to inflammation and diarrhea amongst other conditions[144]. This is well-known in *Clostridioides difficile* infection that may arise from repeated antibiotics usage[135] while fecal microbiota transplants from healthy individuals have been proven highly effective in treating this condition[145].

For most disorders, however, it is unclear whether dysbiosis is the cause or effect of the associated disorder, or whether a bidirectional relationship exists[146]. For example, while certain gut bacteria can influence cortisol release and stress response in

mice[126,147] and humans[148], chronic stress can result in disruption of the gut microbiota[149]. To better understand the egg or chicken dilemma of gut microbiota, we require a better understanding of the mechanisms through which the gut microbiota interact with diseases. Several pathways exist through which dysbiosis may lead to disease. These include changes to the balance between pro- and anti-inflammatory signals[111,114,138], changes to the endocrine signaling in the body[126,147], or alterations in the production of active metabolites[118,139,150].

Dysbiosis, leaky gut, and inflammation

One pathway through which gut microbiota dysbiosis can lead to disease is the disruption of the intestinal epithelial barrier function. Normally, the intestinal epithelium forms an effective barrier, that relies on tight junction molecules to produce a strong linkage between the individual intestinal epithelium cells[110,151]. These interlinked epithelial cells facilitate the absorption of important nutrients, while at the same time preventing antigens and bacteria from entering the body[110,151]. Under normal conditions, intestinal epithelial cells are separated from gut bacteria by two mucin layers, produced by intestinal goblet cells[152]. A disruption of this intestinal barrier and the subsequently increased permeability is termed "leaky gut", and can allow the passage of bacterial products from the intestinal lumen to the body[149,153–156].

Leaky gut may arise from several conditions. These include stress-related cortisol release[154], immunodeficiency in the gastrointestinal mucosa[156], as well as celiac disease following gluten intake[157]. Based on the role of a healthy gut microbiota in maintaining the gastrointestinal epithelium, dysbiosis has been implicated in disruption of the intestinal barrier[110,158,159]. Contact between gut bacteria and the intestinal epithelial cells can initiate increased production of zonulin[160], a protein that increases the permeability of the intestinal barrier through direct regulation of the tight junctions[161]. Furthermore, as demonstrated by Hsiao *et al*.[162], oral treatment with the common gut bacteria *Bacteroides fragilis* are capable of restoring intestinal barrier integrity, further showing how the healthy gut microbiota are important in maintaining the intestinal barrier. Increased intestinal permeability can allow entry of antigens into the bloodstream[151]. Indeed, translocation of the strongly gram-negative endotoxin lipopolysaccharide (LPS) from the gastrointestinal tract into the bloodstream has been demonstrated in several conditions[149,153–156]. The body reacts to LPS through a strong immune response[163], which may explain the link between leaky gut and inflammation, which has been described in previous studies[149,155,162].

1.3 GUT MICROBIOTA IN ADHD AND ASD

1.3.1 INDICATIONS OF GUT MICROBIOTA INVOLVEMENT IN ADHD AND ASD

The observation of the involvement of gut microbiota in neurodevelopment has led to an interest in the role of the gut microbiota in the neurodevelopmental disorders ADHD and ASD[164]. For both disorders, gastrointestinal problems are common[165–169], with one study reporting that 70 % of included children with ASD had a history of gastrointestinal problems compared to 28 % of non-affected children[169]. This points to an involvement of components of the gastrointestinal system in individuals with ADHD and ASD. The association between gastrointestinal problems and ADHD is not well understood, and it is unclear whether gastrointestinal problems are directly associated with the pathophysiology of ADHD or whether they represent differences in diet and behavior[165]. In comparison, children with both ASD and gastrointestinal problems are reported to have more severe behavioral symptoms[168,170] and are less responsive to the pharmacological treatment of aggression or co-occurring hyperactivity[168]. As suggested by Margolis *et al*.[171], the connection between ASD and gastrointestinal problems may be mediated through the mutation of serotonin transporters. An experiment on mice reported that a specific variant of the serotonin-reuptake transporter located in both the brain and the gastrointestinal enterocytes could elicit both ASD-like symptoms, as well as several gastrointestinal problems, including constipation. This is in agreement with a previous study reporting that mutations in this gene increased the susceptibility to ASD[172], which further underpins the association between gastrointestinal functionality and behavioral symptoms in ASD.

In a small study, Pärrty *et al*.[173] observed that children diagnosed with ADHD tended to have a lower relative abundance of *Streptococcus* during infancy. More promising, they showed that early life supplementation with the probiotic *Lactobacillus* had a protective effect against ADHD in later life. Furthermore, while the overall outcome of dietary intervention in ADHD has been inconsistent, it has proven effective in reducing behavioral symptoms in a subgroup of children with the disorder[174]. To generate more knowledge on the link between gut microbiota and ADHD, Tengeler *et al*.[175] transplanted fecal material from participants with ADHD or non-affected controls into germ-free mice. Following this, significant differences in the gut microbiota between the two groups of mice were observed. The mice that received feces from participants with ADHD had an increased relative abundance of several genera belonging to the *Lachnospiraceae* family compared to mice that received feces from non-affected controls. Conversely, the relative abundance of members of the Proteobacteria and Cyanobacteria phyla, as well as the Eubacteriaceae and Ruminococcaceae families, were reduced in ADHD mice. Furthermore, mice that had received feces derived from participants with ADHD had significantly higher anxiety

compared to mice that received feces from controls. Importantly, this behavior was positively correlated with the relative abundance of *Anaerostipes*[175].

More is known concerning gut microbiota in ASD. The first indications of the role of gut microbiota in ASD came from a study by Sandler *et al.*[176] that sought to use oral vancomycin to treat diarrhea in children with ASD. Surprisingly, they observed that several of the children experienced short-term improvements in their behavioral symptoms following treatment. These improvements deteriorated within two weeks of discontinuation of treatment[176]. This sparked an interest in the role of intestinal bacteria in ASD. Soon after, studies reported higher bacteria counts of *Clostridium* species in the feces of children with ASD[177], which were confirmed by other studies utilizing quantitative polymerase chain reaction (PCR)[178], and fluorescence *in situ* hybridization[179]. To investigate whether gut microbiota variations were a cause or effect of ASD, Sharon *et al.*[180] transplanted fecal samples from children with ASD into germ-free mice. Mice that received feces from children with ASD displayed increased repetitive behavior and decreased locomotion and communication compared to mice receiving feces from controls. Furthermore, the gut microbiota of the two groups of mice differed significantly, driven by several bacterial families[180]. Based on this, Kang *et al.* [181] conducted a clinical trial using microbiota transfer therapy from healthy donors to children with ASD. Here, they observed significant improvements both in behavioral symptoms, as well as in gastrointestinal problems[181], all of which were maintained in a follow-up study two years later[182].

1.3.2 GUT-BRAIN AXIS MECHANISMS AFFECTED IN ADHD AND ASD

Gut microbiota can interact with brain function through several pathways included in the gut-brain axis. These include the production of neuroactive metabolites, endocrine signaling, and immune stimulation. The exact pathways involved are not clear, however as will be discussed below, several of the pathways are affected in ADHD and ASD, indicating that the gut-brain axis is indeed involved in the disorders.

Psychoactive metabolites and neuroendocrine signaling

The production of microbial metabolites is not well documented for ADHD. Conversely, Gevi *et al.*[183] demonstrated that children with ASD possessed different concentrations of several urine metabolites involved in tryptophan metabolism, compared to non-affected controls. Tryptophan is an essential amino acid involved in several metabolic pathways. These include synthesis of serotonin, but also bacterial and host-cell mediated digestion[184]. In children with ASD, more metabolites were observed involved in bacterial degradation of tryptophan, whereas the metabolites of

the pathway involved in serotonin synthesis, were reduced[183]. Sharon *et al*.[180] further demonstrated that mice that received fecal transplantation from children with ASD developed variations in several microbial metabolites besides ASD-associated behavioral alterations. In particular, a lower level of the weak GABA receptor agonists 5-aminovaleric acid and taurine were observed in mice receiving feces from children with ASD. To test the effects of these agonists on ASD-like behavior in mice, 5-aminovaleric acid and taurine were administered to a mouse model of ASD. Intriguingly, the continuous administration of these metabolites reduced repetitive behavior and increased social interaction in these mice[180]. This is consistent with earlier observations by Hsiao *et al*.[162], who reported that transplant of metabolites, deficient from a mouse model of ASD, could elicit behavioral modification in naïve mice. Overall, this points to an important role of gut microbiota and its metabolites in the pathology of ASD.

Endocrine disturbances in the HPA axis have been reported for both ADHD[185–187] and ASD[188–190]. For ADHD, a lower baseline cortisol concentration was reported[185,187], whereas, in comparison, no obvious differences were observed for baseline cortisol in ASD[188,189]. In response to stress, children with ADHD had a reduced cortisol response[187], whereas children with ASD had an excessive cortisol response[189]. Wang *et al*.[190] reported that fecal cortisol concentration was negatively correlated to the total abundance of *Clostridium botulinum* and *Eggerthella lenta*. Overall, these observations show that the HPA axis is involved in the pathology of ADHD and ASD. At least for ASD, gut microbiota may be involved in this interaction.

Leaky gut and inflammation in ADHD and ASD

As previously described, changes in intestinal permeability have been linked with gut microbiota-related disorders. Indeed, indications of leaky gut have been reported for individuals with ADHD[191] and ASD[163,192] Children with ADHD were shown to have a higher concentration of serum zonulin, which was positively correlated with ADHD behavioral symptoms. This indicates that a disrupted intestinal barrier is directly related to behavioral symptoms in ADHD[191]. Thus, the presence of increased intestinal permeability in ADHD and ASD might explain the high frequency of immunological diseases observed in the two disorders. This includes autoimmune disorders, especially asthma[193–196] and allergies[195,197]. It is, however, worth noting that the observed high frequency of allergies and asthma in the two disorders might reflect the increased health surveillance for children diagnosed with ADHD or ASD, with large variations observed for studies investigating the presence of autoimmune disorders in ADHD[194,198].

A shift in the balance between pro- and anti-inflammatory cytokines towards inflammation has been observed in ADHD[199]. This was further investigated by Oades

et al.[200] who reported that children with ADHD had higher plasma concentrations of IL-13 and IL-16 compared to matched non-affected controls. Interestingly, these cytokines were positively associated with higher severity of inattention and hyperactivity, respectively, suggesting that the immune variations are directly associated with ADHD rather than just being an incidental finding.

Similarly, several studies have reported that children with ASD possess a higher plasma concentration of pro-inflammatory cytokines, including IL-1β[192,201], TNF-α, TGF-β[202,203], and Eotaxin[203]. The increased Eotaxin concentration was negatively correlated with social functions, suggesting that the inflammatory state might be directly correlated with the affective symptoms in ASD[203]. This observation is supported by two studies that observed brain-specific autoantibodies in the blood of children with ASD[196,204], as well as by the finding of neuroinflammation in the brains of deceased individuals with ASD[205].

Despite these findings, the scale of inflammation among children with ADHD and ASD is not clear, while the cause and effects of the pro-inflammatory state are unknown.

CHAPTER 2.
AIMS

As described in the background section, there are several indications of the role of gut microbiota in ADHD and ASD. While genetics is a major component in the disorders, environmental effects have also been implicated. These include the effects of diet and inflammation. Studies within the last decade have revealed a role of gut microbiota in several neural and psychiatric disorders, as well as in normal neurodevelopment. This has led to an interest in the role of the gut microbiota in ADHD and ASD. Multiple factors involved in the gut-brain axis have been implicated in the disorders, including afferent signals from the gut to the brain, microbiota-dependent disturbances in brain neurotransmitters, and changes in the neuroendocrine and immune system. Finally, experiences from animal studies have shown that gut microbiota, derived from children with either ADHD or ASD, can introduce specific behavioral modifications in mice, indicating that gut microbiota may directly influence symptoms in these disorders. This is further supported by the beneficial effects on behavioral symptoms with dietary interventions for children with ADHD, as well as fecal microbiota transplants for individuals with ASD. Despite a clear overlap between the disorders, the gut microbiota of individuals with ADHD and ASD have not been investigated in parallel.

The overall hypothesis of this study is that children with ADHD and/or ASD possess a distinctively different gut microbiota signature from non-affected children. Based on the overlapping symptoms and hereditary, similarities will be observed for the two disorders. To investigate this, three studies were planned:

Study I:

Studies investigating gut microbiota are susceptible to changes occurring as a result of improper storage. Thus, evaluation of storage methodology is a prerequisite for clinical outpatient microbiota study validity. To facilitate this purpose, these storage methods need to maintain the gut microbiota signatures of the individual samples and be suitable for home sampling by untrained study participants.

Therefore, the aim of study I was to investigate and compare how different methodologies for the storage of fecal samples influence the resulting gut microbiota alpha- and beta-diversity.

Study II:

Although previous studies have provided indications for the involvement of the gut microbiota in ADHD and ASD separately, an overview of the association between microbiota and the disorders are lacking. Thus, before performing a clinical study, it was important to obtain an overview of which gut microbiota had previously been implicated in ADHD and ASD, results from previous studies need to be compared.

The aim of study II was therefore, to systematically summarize and compare studies investigating gut microbiota in individuals with either ADHD or ASD to investigate whether these disorders were associated with distinct gut microbiota signatures. A secondary aim was to investigate whether there was evidence for shared gut microbiota variations in individuals with ADHD and ASD.

Study III:

ADHD and ASD have several features in common, including gastrointestinal comorbidities, several genetic overlaps, and a shared hereditability. Furthermore, increased intestinal permeability has been indicated for both disorders. Together, this suggests that the two disorders are likely to share gut microbiota variations. However, the gut microbiota of ADHD and ASD has not previously been analyzed in parallel. Thus, future studies are needed to investigate the role of the gut microbiota in ADHD and ASD together.

As a result, the aim of study III was to investigate whether individuals with ADHD and/or ASD shared gut microbiota variations and to describe the gut microbiota associated with these disorders. A secondary aim was to investigate whether gut microbiota in individuals with ADHD and/or ASD were associated with changes in intestinal permeability.

CHAPTER 3.
MATERIALS AND METHODS

In the following section, the methods used for the individual studies are described and explained. This includes the recruitment of study participants, laboratory procedures, and statistical tests. More information is available in the original papers at the end of this Ph.D. thesis.

3.1 STUDY I: METHODS FOR STORING FECAL SAMPLES

3.1.1 STUDY DESIGN

Study I was designed as a methodologic study and intended to investigate whether the methodology used for the collection and storage of fecal samples affected the gut microbiota composition as evaluated using gene sequencing. Primary outcomes included variations in bacterial alpha- and beta-diversity between study participants, as well as the presence of differentially abundant bacterial taxa. Secondary outcomes included differences in the bacterial yield and integrity.

3.1.2 STUDY PARTICIPANTS

For study I, three healthy donors younger than 18 years, were recruited through the Centre for Clinical Research, North Denmark Regional Hospital. Parents of the study participants were thoroughly informed of the procedure and purpose of the study before partaking. Exclusion criteria included parental reports of active treatment with antibiotics due to previous studies reporting strong effects of antibiotic usage on gut microbiota composition[96,135,206].

3.1.3 SAMPLE COLLECTION AND STORAGE

Fecal samples were collected by each study participant using a plastic collection kit, and brought to the Centre for Clinical Research, North Denmark Regional Hospital, within one hour following delivery. Upon receival at the laboratory, the fecal material from each of the donors was either immediately frozen at -80 °C, frozen in a domestic freezer at -20 °C, or placed in one of three different storage buffers at either room

temperature or 4 °C. All conditions were run in triplicates from each donor, yielding a total of 45 fecal replicates per donor. For each replicate, 200 ± 50 mg feces were used. The following storage conditions were utilized, resulting in a total of 15 setups:

- PSP buffer at room temperature for 24 and 72 hours
- PSP buffer at 4 °C for 24 and 72 hours
- DNA/RNA shield at room temperature for 24 and 72 hours
- DNA/RNA shield at 4 °C for 24 and 72 hours
- RNA*later*® at room temperature for 24 and 72 hours
- RNA*later*® at 4 °C for 24 and 72 hours
- Domestic freezer at -20 °C for 24 and 72 hours.
- Freezer at -80 °C for at least 72 hours.

All samples were moved to -80 °C storage for a minimum of 24 hours at the end of the storage period. This was intended to prevent artifacts, that may have arisen from freezing, from affecting the results[207].

3.1.4 DNA EXTRACTION AND QUALITY ASSESSMENT

DNA sequencing is dependent on high-quality input DNA. As such, proper extraction protocols are essential. Two steps are involved in the extraction of bacterial DNA: Initial cell lysis to release the DNA from the cells, and isolation of DNA from the cellular debris[208]. Bacterial DNA is found freely in the cytoplasm in a region termed the nucleoid[209]. Accessing this bacterial DNA is, however, complicated by the presence of a rigid cell wall surrounding the bacteria, which is especially thick for gram-positive bacteria. Effective disruption of this cell wall is an essential element of cell lysis in microbiota studies since insufficient disruption can prevent the correct assessment of the composition of gram-positive bacteria[208,210]. Several approaches have been utilized with varying effects to facilitate bacterial cell lysis during DNA extraction, including thermal, chemical, enzymatic[208], or mechanical disruption methods[210].

In this study, DNA was extracted from each fecal sample using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. To facilitate cell wall disruption, four minutes of bead-beating at 30 Hz on a TissueLyzer LT (QIAGEN) were added at the beginning of the DNA extraction protocol[210]. Following bead-beating, the subsequent DNA extraction was automated on a QIAcube® (QIAGEN). The resulting DNA yield was assessed using the Qubit™ HS assay (Thermo Fisher Scientific), while DNA purity were assessed using the ratio of absorbance at 260 and 280 nm at a Nanodrop™ Lite (Thermo Fisher Scientific). While bead-beating has been shown to improve DNA yield from gram-positive bacteria, excessive bead-beating has been shown to lead to fragmentation of DNA samples[210].

This was evaluated using agarose gel electrophoresis where we expect to see a smear of DNA across the higher DNA length based on previous reports by Albertsen *et al*[210].

3.1.5 16S RRNA GENE SEQUENCING OF BACTERIAL DNA

Sequencing was utilized to discern the bacterial composition in fecal samples. This technique allows analysis of either all microbial genomes within the samples (whole genome sequencing) or a marker gene (amplicon sequencing)[211,212]. While whole-genome sequencing can be preferable in some cases due to providing information on bacterial function, the higher cost, as well as a more complex data analysis process causes many studies to utilize marker gene analysis when investigating microbiota. The most commonly used marker gene for microbiota studies is the 16S ribosomal ribonucleic acid (rRNA) gene[211]. Ribosomes exist in all living organisms[213] and consist of a combination of noncoding rRNA molecules and proteins. Together, they form a structure that enables the translation of messenger RNA into proteins. Due to the importance of a correct secondary structure of these rRNA molecules, they tend to be highly conserved although variations exist within specific taxonomic domains. Notably, all prokaryotes (bacteria and archaea) contain the 16S variant of rRNA[214]. The 16S rRNA gene consists of nine hypervariable regions scattered amongst several highly conserved regions[215]. These highly conserved regions allow selective targeting of all 16S rRNA genes in a sample[213], while the hypervariable regions allow the identification of individual bacterial entities[215]. The choice of the target region can lead to variations in the bacterial specificity of the 16S rRNA gene sequencing[210,215].

In this study, the bacterial composition of fecal samples was assessed using Illumina MiSeq-based 16S rRNA gene sequencing targeting the V4 hypervariable region. Briefly, 10 ng of extracted bacterial DNA was used as the input from each sample. Two subsequent PCRs were performed. The first PCR selectively amplified the target sequence and attached adaptors using tailed primers specifically targeting the V4 region of the 16S rRNA gene (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT[216]). Following purification, a second PCR was performed that facilitated the attachment of barcoded adapters to enable multiplexing. After a final purification step, the purified sequencing libraries were pair-end sequenced (2x301 bp) on a MiSeq (Illumina, USA), with 20 % PhiX control library added to estimate error rate. A positive control obtained from an anaerobic digester system, and a negative control, consisting of nuclease-free water were sequenced together with the samples.

3.1.6 BIOINFORMATICS

16S rRNA gene sequencing results in a complex dataset that needs to be processed to provide meaningful information on DNA microbiota composition. Specifically, reads need to be demultiplexed, quality filtered, and have taxonomy assigned. The individual steps are roughly summarized in figure 4.

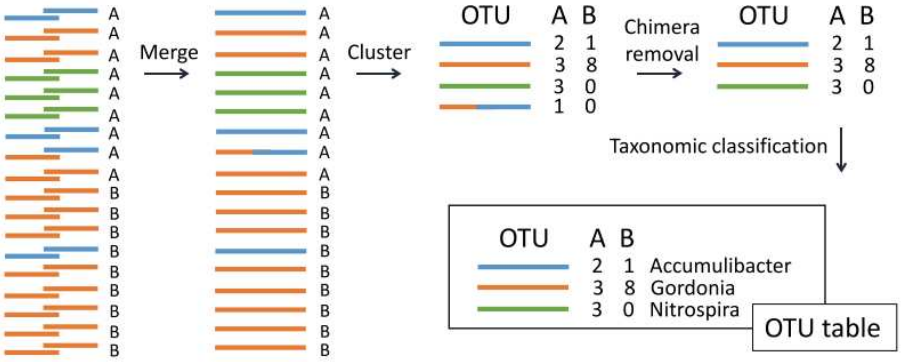

Figure 4. *The different steps involved in 16S rRNA gene-based bioinformatics processing. Chimeras refer to erroneously merged sequences, while OTU refers to operational taxonomic units, a term distinguishing individual bacterial entities following the clustering of sequences with at least 97 % similarity (printed with permission from Loosdrecht et al.* [217]*).*

Several different platforms exist that enable these analyses, including UPARSE[218], QIIME2[219], MOTHUR[220], or Bioconductor[221]. These pipelines are all accepted and widely used, although small variations exist in the relative abundance of individual bacteria obtained in samples when using different pipelines[222]. Briefly, the pipelines consist of the following steps.

1. During preprocessing, sequences are assigned to their respective biological samples, primer sequences are filtered, and low-quality sequences are removed based on the Phred scores (usually below 20)[217,223,224]. For paired-end sequencing, the two reads (forward and reverse) are normally merged into a single sequence, although a low-quality reverse read is common and can make it beneficial to only utilize the forward reads at times[217].
2. During sequencing, small errors may be introduced. For Illumina-based sequencing an estimated error rate of approximately 0.1 % errors per nucleotide is expected[225]. Although small, a large number of nucleotides are analyzed altogether across all sequences. Thus, these errors will add up, leading to minor variations in the final sequences, which can be mistakenly identified as unique bacteria[211]. Thus, these sequencing errors need to be addressed. Traditionally this is handled by clustering sequences that are

within 97 % similarity as individual bacterial entities. These entities are termed operational taxonomic units (OTUs)[223] as described in figure 4.

3. Finally, the bacterial taxonomy can be assigned to the individual OTUs by aligning the representative sequences with established databases for human microbiota like SILVA[226], RDP[227], or Greengenes[228]. This is typically performed using a trained machine-learning algorithm to identify the closest match between the reference and the database[229,230].

For study I on methodology, we utilized a modified UPARSE-based approach[218]. Briefly, quality filtering was performed using the Trimmomatic software v 0.32[231] to remove primers and PhiX sequences, trim the 3' when they fell below a Phred score of 20 (1 % error rate), as well as truncating all reads to the first 250 bp, discarding reads shorter than this. Due to the poor quality of reverse reads, only forward reads were utilized after this step. The resulting sequences were demultiplexed and sequences with >97 % similarity were clustered as OTUs. Finally, taxonomy was assigned to all sequences using the naïve Bayesian RDP classifier[230] as implemented in QIIME[232] to align sequences to the SILVA-derived database MiDAS v. 1.20[233].

3.1.7 STATISTICS

Data analysis for study I was performed using R version 3.4.3 (https://www.r-project.org/) through the Rstudio IDE (http://www.rstudio.com/), as well as Microsoft Office Excell 2013 (Microsoft, USA). The data analyzed involved data on gut microbiota, as well as yield, purity, and integrity of extracted DNA.

For microbiota data, the bioinformatics pipeline described above normally results in a large OTU table. Thus, correct interpretation of the data requires the use of several analyses, with results typically expressed as alpha- and beta-diversity. Alpha-diversity describes variation within the individual samples and includes the observation of bacterial richness and diversity[211]. Bacterial richness indicates the total number of unique OTUs observed within samples[234,235] and bacterial diversity describes the distribution of different OTUs within a sample and can be expressed using Shannon index[236]. While alpha-diversity is used to look at bacterial variability within the individual samples, beta-diversity is used to provide a measure of the similarity and dissimilarity between samples[211]. This requires calculating a distance or dissimilarity metrics representing differences in bacterial composition between samples, which can then be analyzed and visualized using ordination methods like principal component analysis (PCA)[237].

For study I, alpha- and beta-diversity was assessed using the ampvis v. 1.27.0 package[238]. Alpha-diversity was expressed as the number of unique OTUs (OTU richness) and Shannon index, whereas beta-diversity was expressed using Hellinger

transformed OTU abundances and visualized using Euclidean distance in PCA plots. Both OTU richness, Shannon Index, DNA yield, and DNA purity were expressed as metric variables. To determine whether to use parametric or non-parametric tests, we assessed distribution using the Shapiro-Wilks test[239] and variance using Bartlet's test[240]. Normally distributed data were visualized as mean values ± standard deviation and compared using one-way ANOVA with Tukey's post hoc test. Non-parametric data were conversely visualized as median value ± quartiles, and significance was tested using Kruskal-Wallis tests with Dunn's post hoc test[241] and Benjamini-Hochberg's procedure to adjust for a false discovery rate with multiple comparisons[242]. For all tests, the null hypothesis (no difference) was rejected if p-value or adjusted p-value is < 0.05.

3.1.8 ETHICAL CONSIDERATIONS

Since all study participants were anonymized, the North Denmark Regional Ethical Committee waived the requirement for ethical approval for study I in accordance with the Danish Committees Act on Regional Scientific Ethical Committees.

Due to the anonymization, we did not expect any ethical issues to arise during this study although we expected that fecal collection might be unpleasant for some participants. One of the goals of this study was, however, to make observations on the optimal method for feces collection for future studies, and thus, this consideration was a feature of the study.

3.2 STUDY II: SYSTEMATIC LITERATURE REVIEW.

3.2.1 STUDY DESIGN

Study II was designed as a systematic literature review. The purpose of this study was to investigate and compare previous studies looking at gut microbiota in humans with either ADHD or ASD to determine whether these disorders possessed disease-specific gut microbiota signatures. The primary outcomes were gut microbiota alpha- and beta-diversity, as well as measures of significantly different bacteria between the cases and controls. Secondary outcomes included differences and similarities in methodology and demographics.

3.2.1 SEARCH PROTOCOL

This systematic literature review was performed according to the principles described by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement[243], and the protocol was registered at PROSPERO under ID number CRD42018111458. The databases PubMed and Embase were searched prior to July 22nd, 2019, using the search string described in table 1

Search terms (Vertical lines divided by " OR")	**Horizontal lines divided by "AND"**	
	Cases	**Outcome**
	Neurodevelopmental disorders[MESH] Attention Deficit Disorder*[Text Word] Attention Deficit Hyperactivity Disorder[Text Word] ADHD[Text Word] ADD[Text Word] Autism[text word] "Autism Spectrum Disorder"[MESH] Neurodevelopmental*[text word] Neurodevelopmental disorder[MESH]	(Microbiology[MESH] OR Microbiology[Subheading] OR Microbiology[Text Word]) AND (Feces"[MESH] OR Gastrointestinal Tract[MESH]) Gastrointestinal Microbiome[MESH] Gastrointestinal Microbiome*[text word] Gastrointestinal Microbiot*[text word] Gut microbiot*[text word] Gut microbiome*[text word] Intestinal Microbiot*[text word] Intestinal Microbiome*[text word].

Table 1. Overview of s*earch strings used for the systematic review. Modified and printed with permission from Paper II of the PhD thesis*[244].

The following inclusion and exclusion criteria were used.

Inclusion criteria

- The study must be performed in humans diagnosed with either ADHD or ASD as defined by the DSM-IV, DSM-5, or ICD-10 criteria.
- The study must have characterized the complete gut microbiota using fecal samples. Fecal samples are considered a surrogate marker of gut microbiota,

and studies that investigate this in different gastrointestinal regions (e.g. using mucosal biopsies) are known to yield different results[75], and thus, will provide issues during comparisons.
- A control group needed to be included, consisting of humans not diagnosed with either ADHD or ASD.
- The article should be written in English or Danish.

Exclusion criteria

- Studies with less than 10 study participants.
- Studies focused on comorbidities other than ADHD or ASD.
- Studies focused on the effects of intervention against gut microbiota without a baseline measure.

3.2.2 STUDY SELECTION AND DATA EXTRACTION

Following the systematic search, all identified papers were transferred to Mendeley (https://www.mendeley.com/) for the removal of duplicate studies. The remaining studies were transferred to SyRF (http://syrf.org.uk/) for title and abstract screen, which was performed by two independent reviewers. Finally, the complete papers of the remaining studies were read, and the following information were extracted to an excel document (available as a supplement for study II[244]): demographics, methodology, gut microbiota alpha- and beta-diversity, as well as significantly different bacteria between cases and controls.

3.2.3 NEWCASTLE-OTTAWA SCALE-BASED QUALITY ASSESSMENT

The quality of all included studies was assessed using the Newcastle-Ottawa Scale (NOS) for case-control studies[245]. This scale rates studies from 0-10 stars based on three distinct categories:

1. *Selection of cases and controls (four stars)*:
   Stars are given for the selection of representative cases and controls, as well as for whether these are adequately described.
2. *Comparability of cases and controls (two stars)*:
   Two stars are awarded for matching cases and controls for at least two features. These can include age, gender distribution, etc.

3. *Assessment of exposure (microbiota) and outcome (diagnosis, four stars)*: Stars are awarded for describing both how the diagnosis was applied, as well as how the gut microbiota was assessed. While theoretically, four stars could be obtained, this would require a description of exactly how the gut microbiota and neurodevelopmental disorder might be related. Thus, in practice, only two stars were given here.

Based on their score, the quality of the included studies was described as either poor (0-5 stars), moderate (6-7 stars), or high (8-10 stars).

3.3 STUDY III: GUT MICROBIOTA IN CHILDREN WITH ADHD OR ASD

3.3.1 STUDY DESIGN

Study III was a case-control study designed to investigate and compare the gut microbiota of children with ADHD and/or ASD compared to children without these disorders. Primary outcome measures included changes in bacterial alpha- and beta-diversity between study participants, as well as the presence of differentially abundant bacterial taxa. These differences included both differences between cases compared to controls, as well as similarities between children with ADHD and/or ASD. Secondary outcomes included investigating whether gastrointestinal permeability differed between diagnostic groups. Other secondary outcomes include similarities in gut microbiota between siblings and non-related controls, as well as whether gut microbiota variations between diagnostic groups were correlated with changes in gastrointestinal symptoms or differences in clinical features.

3.3.2 STUDY PARTICIPANTS

For study III, children aged 5-17 years diagnosed with either ADHD, ASD, or comorbid ADHD/ASD were recruited as cases. As controls, siblings to the patient groups or non-affected, non-related controls were recruited. Study participants were recruited at the Department for Child and Adolescent Psychiatry at Aalborg University, as well as through social media for the hospital and patient organizations.

Inclusion criteria were children aged 5-17 years, with both ages included. Cases consisted of children diagnosed with ADHD, ASD, or comorbid ADHD/ASD according to the ICD-10[6]. All diagnoses were made before inclusion in the study at a multidisciplinary clinical conference. Children with ADHD were defined according to the ICD-10 diagnostic codes F90.0, F90.1, or F98.8c, with the diagnosis being

applied if children were above the clinical cut-off level for the ADHD rating scale (ADHD-RS)[246]. The cut-off level was defined based on normative data from Danish school children[247]. Similarly, children with ASD were diagnosed with the ICD-10 diagnostic codes F84.0, F84.1, F84.10, F84.11, F84.12, F84.5, or F84.8 if children were above a calibrated severity score of 3[248] for the Autism Diagnostic Observation Schedule 2nd edition (ADOS2[249]). Controls consisted of non-related children without neurodevelopmental disorders, as well as non-affected siblings to the three patient groups. Per the Danish Committees Act on Regional Scientific Ethical Committees, the controls were not screened for ADHD and ASD. Instead, the absence of neurodevelopmental disorders was assessed based on parent interviews.

Exclusion criteria were active treatment with antipsychotic medication (Risperidone, Aripiprazole, and Quetiapine), selective serotonin receptor reuptake inhibitors, or treatment with antibiotics within three months prior to inclusion. These groups of drugs have been reported to have antimicrobial effects, and thus, it was decided to remove them[97,206,250]. Furthermore, children with one of the following diagnoses according to the ICD-10 criteria were removed from the studies: substance abuse, diagnosis of a manic episode, bipolar affective disorder, eating disorder, organic psychiatric disorder, schizophrenia, and other psychotic disorders. Several of these diagnoses have been suspected to be associated with gut microbiota variations[142,251–253] or might influence the diagnosis of neurodevelopmental disorder.

3.1.3 CLINICAL AND DEMOGRAPHIC INFORMATION

For study III, several types of clinical information were obtained. These included information on diet, defecation patterns, clinical information on past and current diseases, and the use of medication. This information was obtained through an interview before inclusion, as well as through medical records.

Information on diet was obtained through the initial interview, where parents of the included children were asked to describe the typical diet of the child in general and in the previous week. A restricted diet was defined as a diet that either excluded several common food items or that did not vary between days. Information on defecation patterns, the presence of unspecified minor illnesses (e.g. common cold, headaches, stomach pain, or fever) immediately before sample collection, as well as on the composition of the fecal sample in which microbiota was investigated were obtained using a defecation diary covering the 14 days before sample delivery. In this, the parents of the study participants were asked to describe the daily defecation frequency and consistency according to the Bristol Stool Scale[254], as well as to define the delivered fecal sample based on the same tool. Based on this information, constipation was defined as less than three defecations per week as described by Van den Berg *et*

al[255]. The parents were likewise asked to describe the use of medications, as well as medical issues during this time.

3.3.4 SAMPLE COLLECTION

From all study participants, fecal and blood plasma samples were collected as visualized in figure 5.

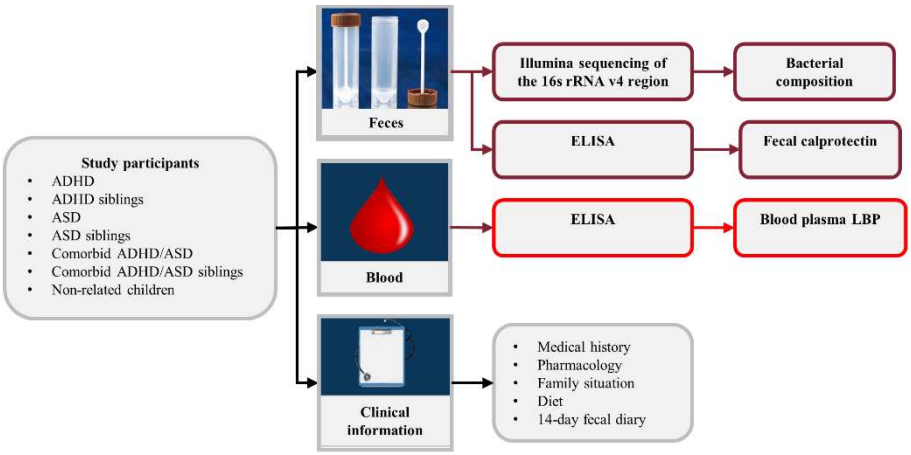

Figure 5: *A visualization of the design for study III.*

Fecal samples were collected at the home of study participants, using written, oral, and video guides to facilitate proper sample handling. The parents of study participants were instructed to immediately store the fecal samples at -20 °C in a home freezer, using equipment provided. Within three days, fecal samples were transported on ice to the Clinical Biochemistry Departments at either Aalborg University Hospital or North Denmark Regional Hospital. Here, blood samples were drawn in Vacuette tubes containing K2-EDTA (Greiner Bio-One GmbH, Austria) by experienced personal. Plasma was produced by centrifuging these blood tubes at 2200*xg* for 10 minutes at 4 °C. Following collection, fecal and plasma samples were aliquoted and placed at -20 °C until delivery to the Centre for Clinical Research within 72 hours, where they were finally stored at -80 °C.

3.3.5 DNA EXTRACTION AND 16S RRNA GENE SEQUENCING

As mentioned for study I, effective DNA extraction is imperative for DNA sequencing. For study III, bacteria DNA was extracted from 250±25 mg fecal sample using QIAamp PowerFecal DNA kit (QIAGEN) automated on a QIAcube (QIAGEN) according to manufacturer's instruction. This kit is a further development of the kit used for study I, with the inclusion of bead-beating and improved buffers. As described by Lim *et al*.[256] the output of QIAamp PowerFecal DNA kit was comparable to the QIAamp DNA Stool Mini kit modified to include bead-beating for gut microbiota studies.

The bacterial composition of the fecal samples was sequenced using Illumina MiSeq sequencing of 16S rRNA gene, targeting the V4 region. 10 ng extracted DNA was used as input for each sample, with library generation and sequencing being performed using two subsequent PCRs. The first PCR was used to amplify the target sequence and attach adaptors, using primers targeting the V4 region of the 16S rRNA gene (515F(Parada): GTGYCAGCMGCCGCGGTAA[257] and 806R(Appril): GGACTACNVGGGTWTCTAAT[258]). Following purification, a second PCR was performed during which barcoded adapters were attached to enable multiplexing. After a final purification step, the sequencing libraries were pair-end sequenced (2x300 bp) on a MiSeq (Illumina), using the MiSeq reagent kit V3 (Illumina). 10 % PhiX control library was added to estimate the error rate.

This protocol is similar to that of study I, but with a few modifications. First, only 10 % PhiX control library was added rather than 20 %. This is, however, not expected to affect the results. Moreover, the 515F-806R[216] primer pair used for study I was replaced with the 515F(Parada)[257]-806R(Appril)[258] primer pair. These modifications correct previous biases towards marine and freshwater bacterial taxa[257,258]. While these bacterial taxa are not considered relevant to this study, these updated primers are comparable to the previous primer set for gut microbiota research. Furthermore, they have been included as the standard primers for the Earth Microbiome Project[259] and thus have been utilized in this study in the interest of standardization of methodologies in microbiota studies.

3.3.6 QIIME2-BASED PROCESSING OF SEQUENCING READS

A different bioinformatics pipeline was utilized for study III compared to study I. Initially, reads were demultiplexed, and PhiX spike-in sequences were filtered using parts of the usearch v11 pipeline[260]. The demultiplexed reads were then processed in the QIIME2 v. 2020.8 software[219]. DADA2[261] was used for primer removal and quality filtering. Forward reads were truncated to 250 bp, while reverse reads were discarded due to their low quality as indicated by their respective Phred scores. While

study I clustered samples based on 97 % similarity to address sequencing errors, this risked missing variations amongst closely related bacteria, which can be critical when attempting to discern variations between different diagnostic groups[262]. The DADA2 algorithm handles this using error prediction models to identify sequencing errors. As a result, unique sequences could be utilized directly as amplicon sequence variations (ASVs) in study III, enabling higher resolution[261]. The taxonomy of the resulting sequences was then assigned using the naïve Bayesian classifier q2-feature-classifer[229] to align the sequences to the SILVA 138 SSU database[226]. To enable comparisons of taxonomic relationships between bacteria in samples, a phylogenic tree was constructed using the fasttree2 algorithm[263].

3.3.7 IMMUNOASSAY-BASED ASSESSMENT OF GASTROINTESTINAL PERMEABILITY

The permeability of the gastrointestinal epithelium of study participants was tested indirectly through the measurement of blood plasma LBP, as well as fecal calprotectin. LBP is a protein that is rapidly produced by the body in response to an increased concentration of the gram-negative specific surface molecule LPS in the blood and is thus used as a highly sensitive indicator of LPS concentration[149,153]. Calprotectin is a protein associated with neutrophil granulocytes[264]. In response to gastrointestinal inflammation, these granulocytes migrate through the gastrointestinal barrier to the lumen. As such, the presence of calprotectin in feces is used as an indicator of increased migration of inflammatory cells through the gastrointestinal barrier, and thus of localized inflammation in the gastrointestinal tract, especially in inflammatory bowel disorders[265].

In this study, plasma LBP was measured using an enzyme-linked immunosorbent assay (ELISA), while fecal calprotectin was detected using the related fluorescent enzyme immunoassay (FEIA). Both immunoassays utilize the highly specific and sensitive antibody-antigen binding to detect their target molecules and are based on the sandwich immunoassay technique. The procedure is summarized in Figure 6 and described in more detail for LBP and calprotectin measures separately.

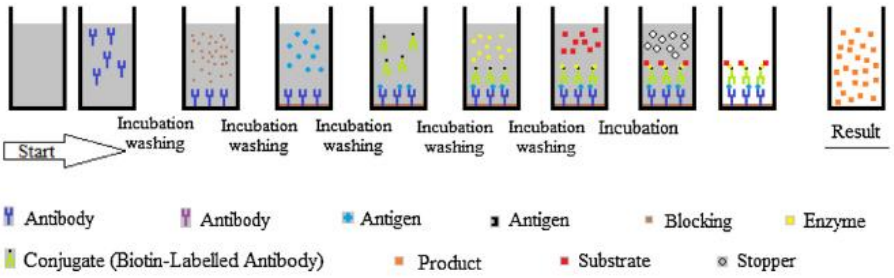

Figure 6: *A visualization of the sandwich immunoassay method, as utilized by both the LBP ELISA and the calprotectin FEIA (modified and printed with permission from Aydin et al.*[266]*).*

LBP was measured in plasma samples using the RayBio® Human LBP ELISA kit (RayBiotech, USA) according to the manufacturer's instructions. All samples were tested in duplicates and all incubation steps were performed at room temperature using an orbital shaker (Thermo Fischer Scientific, USA) set to 150 rpm. Briefly, the procedure was as follows: Plasma samples were diluted 1:1000 in an assay buffer and added to a microbiota that was well precoated with anti-LBP antibodies. In parallel wells, a dilution series was performed with known LBP concentrations ranging from 0.819 – 200 ng/mL. The anti-LBP coating in the wells binds LBP present in the samples. Next, anti-LBP antibodies attached to biotin were added to all wells followed by streptavidin-conjugated horseradish peroxidase (HRP). Anti-LBP antibody binds to the already-bound LBP, while the streptavidin binds strongly to the biotin on the antibody. Next, a substrate (tetramethylbenzidine) was added to all wells. HRP present in the wells starts to oxidize the substrate, producing a green-blue color. The more HRP linked to antibodies in the well, the faster the conversation, and thus, the more colored product are produced[266]. After a set period, the oxidization was stopped through the addition of an acidic stop solution (H_2SO_4). This caused the solution to change color into yellow[266], and the color intensity was measured at 450 nm. A strong color indicated that more substrate were oxidized, and thus, a higher starting concentration of LBP was present[266]. To determine the precise LBP concentration, the measured color intensity was compared to a standard curve generated using a four-parameter logistic regression curve[267] based on color intensity, and the LBP concentration in the standard dilution series.

Fecal calprotectin was analyzed using the EliA™ calprotectin 2 kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Compared to the manual LBP measurement, the fecal calprotectin FEIA was automated on the Phadia 250 instrument (Thermo Fisher Scientific). Briefly, the procedure was as follows: Fecal samples were homogenized with EliA™ Calprotectin 2 extraction buffer (Thermo Fisher Scientific) and diluted 1:200 with sample diluent. The diluted samples were added to the Phadia 250 instrument together with premade calibrator controls ranging

from 3-750 ng/mL. The plates were precoated with anti-calprotectin antibodies. Next, β-galactosidase linked anti-calprotectin antibodies were added to the solution. These antibodies then converted a substrate (4-methylumbelliferyl-β-D-galactoside) into a fluorescent product[268]. Following incubation, the procedure was stopped through the addition of NaOH. The Phadia 250 instrument produced a standard curve based on the calibrator control and used this to calculate the calprotectin concentrations.

3.3.8 STATISTICS

For study III, all statistical tests were performed using R version 4.0.3 (https://www.r-project.org/) through Rstudio IDE (http://www.rstudio.com/). The analyzed data included bacterial alpha- and beta-diversity, demographic data, and ELISA results. For all statistical tests, children with ADHD, ASD, and comorbid ADHD/ASD were compared to non-related controls, the matching sibling group, and each other.

Alpha- and beta-diversity were assessed using the R packages Phyloseq v.1.32.0 and ampvis2 v2.6.6. Alpha-diversity was expressed as the number of unique ASVs (ASV richness), diversity as indicated by Shannon index[236], and evenness as indicated by Pielou's evenness measure that compares diversity with the maximal possible diversity for the given richness[235,269]. Using the generated phylogenic tree, the phylogenic distance was expressed through Faith's phylogenic index[270]. For beta-diversity, a different approach was used compared to study I. While study I utilized Helllinger transformed OTU abundances, as well as the Euclidean distance, this is not always appropriate for studies comparing gut microbiota between diagnostic groups[237]. Instead, the beta-diversity for study III was measured using Bray-Curtis dissimilarity[271], as well as weighted and unweighted UniFrac[272]. Bray-Curtis dissimilarity measures the degree of dissimilarity weighted for the abundance of each ASV[271], while UniFrac measures the total phylogenic distance between the taxa in each sample either weighted for the abundance of ASVs (weighted UniFrac) or calculated based on the presence or absence of taxa (unweighted UniFrac)[272]. The individual measures have distinct advantages and disadvantages, and thus, a combination of these measures was used to facilitate a better description of the gut microbiota of samples[211]. The resulting beta-diversities were visualized using principal-coordinate analysis (PCoA) plots along with 95 % confidence intervals and tested using permutational multivariate analysis of variance (PERMANOVA)[273]. Finally, the bacterial taxa defining the individual diagnostic groups were assessed using the linear discriminant analysis effect size (LEfSe) method. This technique identifies the ASVs that are best at distinguishing the individual diagnostic groups[274].

The distribution of metric data in study III was tested using Shapiro-Wilks test[239], while variance was tested using Bartlett's test[240]. Depending on the distribution, data were compared using either one-way ANOVA with Tukey's post hoc test (parametric

data) or Kruskal-Wallis tests with Dunn's post hoc test using Benjamini-Hochberg's procedure to check for a false discovery rate (non-parametric data)[242]. For all tests, the null hypothesis of no difference was rejected if either the p-value or adjusted p-value was <0.05.

3.3.9 ETHICAL CONSIDERATIONS

The use of human subjects in study III was approved by the North Denmark Regional Ethical Committee (reference: N-20170064) and registered with the Danish Data Protection Agency through the North Denmark Regional Hospital. Parents and legal guardians of study participants were thoroughly informed about the study orally and written before signing an informed consent form.

Potentially ethical issues associated with the study included discomfort during the collection of fecal and blood samples. The collection of fecal samples can potentially be considered uncomfortable by some participants. However, as determined during study I, the technique was fast and non-invasive, while discomfort was minor, and the procedure is quick. Secondly, obtaining blood samples might be slightly painful for the included children. Thus, personnel experienced in drawing blood from children were used for all participants, and less than 1 % of total blood volume was drawn. All studies investigating biological markers in humans, risk accidentally revealing clinically relevant data. This is especially important for calprotectin that is actively used clinically[275]. To deal with this issue, all parents of study participants were asked before inclusion whether they wished to be informed if clinically relevant data were uncovered during the study. Clinically relevant data would be discussed with an experienced clinician before informing parents of study participants.

CHAPTER 4.
RESULTS AND DISCUSSION

The following contains a summary and short discussion of the results of the individual studies included as part of this thesis. A detailed presentation is provided within each paper.

4.1 STUDY I: INTERPERSONAL VARIATIONS IN GUT MICROBIOTA PROFILES SUPERSEDES THE EFFECTS OF DIFFERING FECAL STORAGE CONDITIONS

Aim of study I:

To investigate and compare how different methodologies for the storage of fecal samples influence the resulting gut microbiota alpha- and beta-diversity.

4.1.1 MAIN FINDINGS

Studies examining gut microbiota often rely on home sampling by study participants. While convenient, this also introduces risks of improper storage of the samples, which can favor the growth of certain bacteria following defecation, and thus, potentially alter the microbial composition[276]. As an example, the increased access to oxygen during storage has the potential of favoring aerobic bacteria[277]. This study, therefore, sought to validate methods for storing fecal samples for gut microbiota studies in a way that was feasible for home collection by untrained individuals. To do this, fecal samples were obtained from each of the three healthy donors. From each donor, fecal samples were stored in technical triplicates either in storage buffers at room temperature or 4 °C, frozen at -20 °C for 24 and 72 hours, or as a gold standard, at -80 °C, resulting in a total of 45 replicates from each donor feces.

While variations in bacterial alpha- and beta-diversity were observed for individual donors, the samples were clustered by donor identity rather than storage methodology when comparing beta-diversity as visualized in figure 7. Thus, interpersonal variations superseded any variations introduced by storage methodology. This is consistent with previous studies on the storage of fecal samples for gut microbiota[276,278]. In particular, donor C was observed to be highly different from donors A and B, having a higher relative abundance of an OTU belonging to the order

Gastranaerophilates, as well as an uncultured OTU belonging to the family *Lachnospiraceae*. These differences may arise from differences in genetics[279,280], age[88 281], or lifestyle feature like diet[90,92,282] or exercise[283].

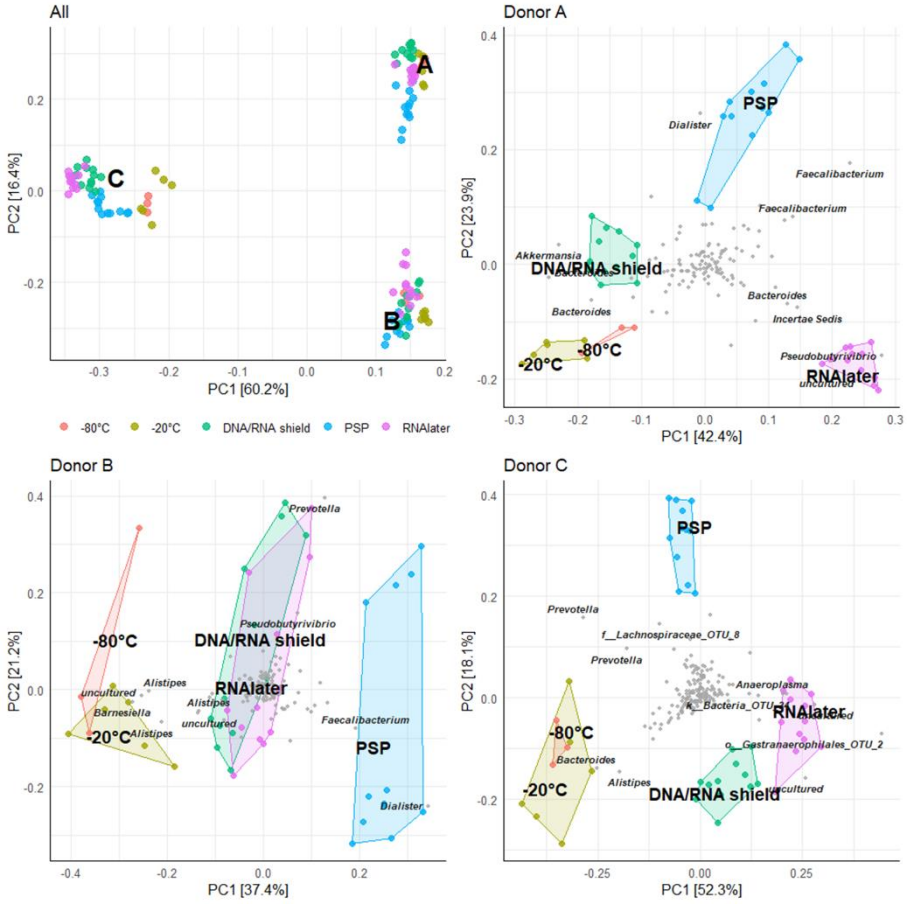

Figure 7: *Differences in beta-diversity introduced by fecal donor and storage conditions for all samples, as well as between storage conditions for individual donors A, B, and C. Clustering was based on Hellinger distance and visualized using PCA plots, with colors specifying individual storage conditions. The bacterial species most identified with individual samples are marked on the PCA plots using grey points (modified with permission from data used in study I of this Ph.D. thesis[284]).*

While all storage conditions could maintain the interpersonal gut microbiota signature making them viable for studies investigating differences between participants, storage did introduce minor variations within donors that may be important in some studies. One example involved the genus *Faecalibacterium* that had a higher relative

abundance in any of the storage buffers compared to samples frozen at -20 °C and -80 °C (figure 8). Conversely, the relative abundance of the genus Prevotella was lower in samples stored in RNA*later* compared to all other storage conditions. These bacterial genera have previously been reported to be susceptible to changes during storage[285–287]. The buffer-associated variations were further underlined when looking at the DNA yield. All storage buffers, except for DNA/RNA shield at room temperature and 4 °C for 24 hours yielded significantly more DNA compared to frozen samples. The storage-related variations were observed to be related to storage methodology rather than the storage duration. Since no differences were observed between the buffers at 24 or 72 hours, this indicates that the effects of the buffer itself introduce these variations rather than changed bacterial growth potential. As similarly suggested by Menke *et al.*[288], the storage-specific variations may be caused by components within the buffers that affect DNA extraction or downstream analyses. The data in this study is, however, not sufficient to reliably answer this. Importantly, storing fecal samples in a home model freezer at -20 °C, maintained the DNA yield, as well as bacteria alpha- and beta-diversity comparable to that of the gold standard, -80 °C. This further confirms the potential of -20 °C storage of fecal samples for gut microbiota[278,286,289].

Finally, since excessive bead-beating may lead to the destruction of DNA, and subsequently prevent identification of low-abundance bacteria[210,290], DNA integrity was assessed using gel electrophoresis. Neither bead-beating nor differences in storage methodology severely affected DNA integrity.

Overall, all storage conditions were able to maintain the donor-specific bacterial signature, as well as providing a good DNA yield and integrity. As a result, all of the investigated storage methodologies were found to be suitable for gut microbiota studies.

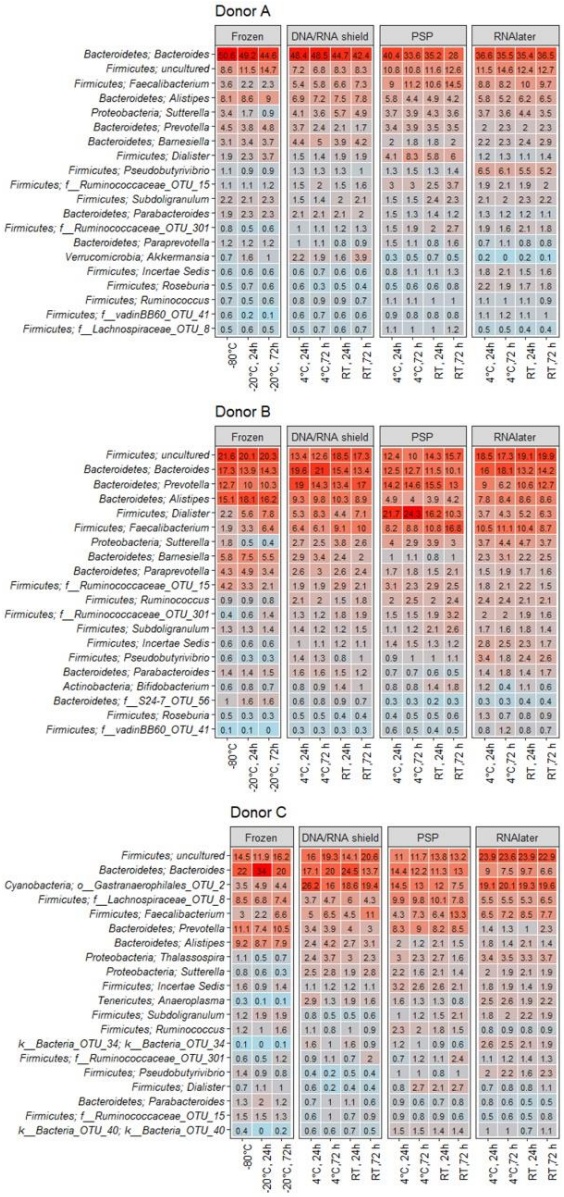

Figure 8: *Differences in the relative abundance of the individual bacteria for donors A, B, and C. Only the 25 most abundant OTUs are displayed. The taxonomy of the individual OTUs is assigned at a genus level, listed alongside the phylum it belongs to. If no genus name was identifiable for an OTUs, the best assignment is shown instead (modified with permission from data used in study I of this Ph.D. thesis*[284]*).*

4.1.2 METHODOLOGICAL CONSIDERATIONS

In this section, challenges of study I are mentioned, and the methodology is discussed.

During the study design, it was decided to go for a low number of donors and instead to focus on a higher number of technical replicates (i.e. several samples from the same overall fecal material). The purpose of this was two-fold: first, it allowed better visualization of the variation in storage conditions alone rather than representing variations between humans. Secondly, it allowed a shorter recruitment period for the study. However, as evident in this study, there is a large degree of interindividual differences in gut microbiota profiles. Previous studies have described at least three distinct overall enterotypes[78], and as a result, we cannot be sure that we have tested the viability of storage for all enterotypes. Indeed, we observed that the genus *Prevotella*, a genus identifying one of the enterotypes, was more susceptible to storage in RNA*later*. It would have been beneficial to have utilized the same number of samples from a larger cohort, however, that would have made the study prohibitively expensive for a methodology study.

While overall gut microbiota signatures were maintained for all storage conditions in this study, the methodology did introduce limitations in detecting minor variations. Gut microbiota were evaluated using amplicon sequencing of the 16S rRNA gene. Although this approach is widely used in studies investigating gut microbiota in disorders[211], it has been shown to be unreliable for species-level identification[291]. Since the main purpose of study I was to test methods for feces collection that were to be used for study III, which also utilized 16S rRNA gene sequencing, this is not considered a major issue. Clustering samples as OTUs rather than applying error prediction algorithms to form ASVs may have masked smaller variations due to the lower resolution of OTUs compared to ASVs[261]. The use of ASVs was, however, not implemented at the department until later during the Ph.D. process. Furthermore, despite misgivings, clustering samples as OTUs is still an accepted technique as evident in the included studies in study II, as well as in newer studies[292–295].

To prevent downstream effects of the storage buffers on the subsequent sequencing[296], it was important to effectively separate the fecal sample from the buffer before DNA extraction[288]. However, we observed that it proved difficult to fully separate RNA*later* from fecal samples, even using centrifugation. Thus, methods relying on storage of fecal samples in RNA*later* need to use DNA extraction methods that can handle remaining buffer.

4.1.3 STRENGTHS AND LIMITATIONS

Certain methodological limitations inherent in this study need to be addressed. To reduce variations, fecal samples from the same donor were taken from the same initial feces. However, as described by Voigt *et al*.[297] the microbial composition can vary across the fecal sample for some donors. To address this, all fecal samples were sampled at the surface of the feces, and close to each other. While this is expected to reduce location-associated variation, this cannot be completely ruled out. Secondly, all samples in this study were eventually frozen at -80 °C, with no freshly extracted samples being analyzed. Thus, potential freezing-induced changes were not investigated. However, in a previous study, Fouhy *et al*.[207] described that freezing samples at -80 °C were effective at preserving the gut microbiota compared to freshly handled fecal samples. Finally, one of the outcome measures in this study consisted of DNA yield following storage. However, it is unclear whether an increased DNA yield represented bacterial growth during storage or represented storage-induced lysis of cellular membranes. Since bacterial yield was measured as total DNA rather than a selective measurement of bacterial DNA (e.g. through quantitative PCR targeting the 16S rRNA gene), it is unclear whether changes in DNA yield represented bacterial or human DNA.

Despite these limitations, the study has strengths as well. While the study population was low, the use of technical replicates and the high number of samples from the same feces allowed a focused investigation of the effects of storage, limiting the bias introduced during sample collection. Secondly, all techniques involved in storing samples utilized in this study were low-cost and compatible for usage by untrained individuals, making them useable for large-scale studies relying on home sampling. Importantly, the -20 °C freezer tested in this study was not a laboratory freezer but rather a standard home model, further showing its usability. Finally, the use of a well-characterized positive control ensured good sequencing coverage.

4.2 STUDY II: GUT MICROBIOTA PROFILES OF AUTISM SPECTRUM DISORDER AND ATTENTION DEFICIT/HYPERACTIVITY DISORDER: A SYSTEMATIC LITERATURE REVIEW.

Aim of study II:

To systematically summarize and compare studies investigating gut microbiota in individuals with ADHD or ASD to investigate whether these disorders were associated with distinct gut microbiota signatures. A secondary aim was to investigate whether there was evidence for shared gut microbiota variations in individuals with ADHD and ASD.

4.2.1 MAIN FINDINGS

Previous studies have reported variations in gut microbiota associated with ADHD or ASD. However, a specific ADHD-or ASD-associated gut microbiota signature has not been established[64,164,298]. In this systematic review, we set out to investigate the gut microbiota described in ADHD and ASD in previous studies, to examine whether specific ADHD- or ASD-related gut microbiota signatures could be described.

Data on methodology, bacterial alpha- and beta-diversity, as well as microbial composition, were extracted from a total of 24 articles. These consisted of four articles investigating the gut microbiota associated with ADHD[299–302], and 20 articles investigating the gut microbiota associated with ASD[170,181,309–318,190,202,303–308]. Of these, four studies (all ASD) received a high NOS score[170,305,308,312], while the remaining studies all received a moderate quality score. The studies investigating ADHD included 114 cases with ADHD, 21 non-affected sibling controls, and 135 non-related controls. Similarly, the studies investigating ASD included 733 cases with ASD, 138 non-affected siblings, and 452 non-related controls. None of the studies included study participants with comorbid ADHD/ASD. Both cases and controls were younger than 18 years of age in all studies, except for a single study investigating ADHD[299].

Conflicting results were obtained when comparing studies investigating gut microbiota in individuals with ADHD as indicated in table 2. Amongst the four studies, only Wang *et al*.[319] observed variations in alpha-diversity between children with ADHD and controls. Two studies reported that the bacterial beta-diversity varied between cases with ADHD and non-related controls[299,300], while the remaining two studies did not observe any differences[301,302]. All four studies were able to identify bacterial taxa that had different relative abundance in ADHD compared to controls although the results were too heterogenous to reliably identify any overall ADHD-related gut microbiota signature. Several of the bacteria with a reduced relative abundance in cases with ADHD were bacteria that have been related to maintaining normal gastrointestinal function[91,320–323]. These included the bacteria *Parabacteroides, Prevotella*[300]*, Faecalibacterium, Dialister*[301], and *Lactobacillus*[302]. One study attempted to investigate how the ADHD-related gut microbiota profile interacted with the gut-brain axis[299]. It was observed that a higher relative abundance of *Bifidobacterium* was associated with increased activity of the enzyme cyclohexadienyl dehydratase[299] that is involved in dopamine metabolism[121,122]. Importantly, the activity of the enzyme was negatively associated with reward anticipation response in the ventral striatum[299]. This further links the gut microbiota with previously observed dopamine depletion in the brain and deficiencies in related reward anticipation response for ADHD[37–39].

Most of the included studies agreed that children with ASD possessed a different gut microbiota composition compared to controls, although no consistent ASD-specific

gut microbiota signature could be identified (Table 2). Some bacterial genera were, however, repeatedly shown to differ in children with ASD compared to non-related controls. These involved a reduced relative abundance of *Bifidobacterium*[181,304,312,314,318], *Coprococcus*[310–312], *Dialister*[314,315,318], *Faecalibacterium*[311,312,314], *Prevotella*[306,310–312], and *Streptococcus*[304,305,312,313,318], as well as an increased relative abundance of *Bacteroides*[303,304,312,318], *Barnesiella*[305,311,312], *Clostridium*[190,306,312,314], and *Roseburia*[304,312,314]. A few of the studies investigated the involvement of the gut-brain axis. As for ADHD cases, the concentration of the dopamine and noradrenaline precursor phenylalanine[121] was reduced in fecal samples of children with ASD in one study[190]. Similarly, the neurotransmitter GABA was reduced in fecal samples in cases with ASD in two studies[311,312], whereas cases with ASD had elevated fecal glutamate concentrations[311]. In agreement with previous observations, this was driven by reduced glutamate metabolism[44,190]. While glutamate is normally important for neural functioning, excessive glutamate concentrations have been demonstrated to induce neural apoptosis[45]. Two studies suggested that these gut microbiota variations might be related to an increased inflammatory state[170,202], whereas the gut microbiota variations observed in children with ASD were associated with changes in the metabolism of nutrients[190,202,304,305,308,312–314]. Too little information was, however, available to reliably conclude on the function of the microbial variations.

The included studies varied widely regarding demographics and methodology, which might explain some of the heterogeneous bacterial findings between studies. A marked geographic difference was observed for ADHD, with studies observing a difference in bacterial beta-diversity all originating in Europe[299,300], whereas those that did not observe any differences all originated in East Asia[301,302]. Geographical variations in gut microbiota have previously been reported[88,93], although it is unclear whether differences in geography or lifestyle might explain these variations. In individuals with ASD, gastrointestinal symptoms, like constipation, were much more common amongst cases compared to controls in several studies. Since transit time heavily impacts the gut microbiota composition[95], these symptoms might explain some of the variations in gut microbiota observed in individuals with ASD. Finally, the studies varied widely regarding storage methodologies, DNA extraction methodology, gene targets, bioinformatic pipelines for 16S sequencing results, as well as the choice of sequence database. All of these have the potential of introducing biases towards or against specific bacteria[210,222,276].

	ASD																				Total		ADHD				Total	
Studies	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	↑	↓	69	70	71	72	↑	↓
α-diversity																												
Richness	↓		↑	↓					↓					↑			↑	↓	↓		3	5				↑	1	0
Diversity			↑	↓			↓		↓			↓	↑		↓	↓	↑	↓			3	7		↓		↑	1	1
β-diversity	D	N	D	N	N	D	N	N	D	D	D	D	D	D	-	D	D	D	-	-	D=12		D	D	N	N	D=2	
Phylum																												
Actinobacteria	↓												↓						↑	↑	2	2	↑				1	0
Bacteroidetes	↑		↑			↓		↓		↓		↑	↑				↑				5	3					0	0
Firmicutes	↓		↓			↑		↑		↑		↓				↓	↓				3	5					0	0
Proteobacteria	↑												↑							↑	3	0					0	0
Genera																												
Actinomyces												↓	↓								0	2					0	0
Bacillus																	↑			↑	2	0					0	0
Bacteroides	↑		↑										↑				↑				4	0		↑			1	0
Barnesiella			↑						↑							↑					3	0					0	0
Bifidobacterium	↓		↓				↓	↑		↓			↓							↑	2	5	↑				1	0
Butyricimonas										↓		↑								↑	2	1					0	0
Clostridium	↓		↑							↑				↑					↑		4	1					0	0
Collinsella	↓		↑			↑															2	1					0	0
Coprococcus			↓	↓					↓				↑								1	3					0	0
Dialister	↓					↓				↓											0	3			↓		0	1
Dorea			↑			↑															2	0					0	0
Enterococcus																↑				↑	2	0					0	0
Escherichia			↓									↓									0	2					0	0
Eubacterium			↓													↓					0	2					0	0
Faecalibacterium			↓						↓	↓			↑								1	3			↓		0	1
Lachnospira			↓										↑					↓			1	2					0	0
Lactobacillus						↑		↑													2	0				↓	0	1
Megaspaera								↑						↑							2	0					0	0
Oscillospira			↓										↑				↑				2	1					0	0
Parabacteroides	↑		↓			↓							↑				↑				3	2		↓			0	1
Prevotella			↓	↓					↓					↓						↑	1	4		↓			0	1
Pseudomonas														↑		↑					2	0					0	0
Roseburia			↑							↑			↑								3	0					0	0
Ruminococcus	↓		↑										↑								2	1					0	0
Streptococcus	↓		↓									↓	↓	↑		↓					1	5					0	0
Sutterella												↑					↑				2	0					0	0
Turicibacter	↓		↓																		0	2					0	0
Veillonella						↓						↓									0	2					0	0

Table 2: *Table depicting the variations in bacterial alpha- and beta-diversity between ADHD and ASD cases, and controls. ↑ and ↓ indicates that the alpha- or beta-diversity or relative abundance of bacteria were higher or lower, respectively, in cases compared to controls. Empty boxes indicate that no difference for this measure was reported. N = no difference in beta-diversity. - = no information. Study number refers to references in Study II. Printed with permission from study II in this PhD*[244].

In conclusion, the systematic review revealed that gut microbiota in individuals with ADHD or ASD differed from that of non-affected controls. The highly varied methodologies and demographics of the individual studies did, however, result in a high degree of variation in the gut microbiota between studies. As a result, a specific ADHD or ASD gut microbiota signature could not be identified. Several of the included studies did indicate that changes might be related to alterations in the interaction between the gut microbiota and the host. Future studies are needed to investigate the interaction between gut microbiota, inflammation, gastrointestinal function, and metabolomics in children with ADHD and/or ASD. Importantly, the data for individuals with ADHD was too diverse to identify overlapping microbial signatures between ADHD and ASD. To overcome variations introduced by differences in methodology, the microbiota of these two disorders should be investigated in parallel.

4.2.2 METHODOLOGICAL CONSIDERATIONS

To investigate the current knowledge on the connection between neurodevelopmental disorders and gut microbiota in an unbiased way, it was decided to conduct a systematic review according to the principles described by PRISMA statement[243]. Below, methodological considerations for certain of the items included herein are listed.

- *Objectives (item 4)*: The main research question used in study II, was "Is the gut microbiota of humans with either ADHD or ASD different from that of controls?". Based on the identified results, as well as other systematic reviews within this field[164,324], this research question was considered fitting.

- *Eligibility criteria (item 6)*: Study characteristics and search terms were presented using the patient-, intervention-, comparison-, and outcome (PICO) statement modified to be appropriate for an observational case-control study. A conscious decision was taken to only include studies that assessed the whole gut microbiota rather than testing selectively for certain bacteria (e.g. using PCR or culture-based techniques). While selective measures could have provided information on individual bacteria, the methodological variations between sequencing studies already complicated data analysis significantly. Including qPCR and culture-based techniques would have made comparisons between studies even more complicated. Despite this, these data might have produced beneficial information for this systematic review.

- *Information sources (item 7)*: Studies were identified in the databases PubMed and Embase. While these two databases are the most widely used[325], some studies may have been missed. This could have been either because they were only available in specialized databases or that they had been improperly indexed. It might have been beneficial to include a specialized database like PsychInfo as suggested by Bramer *et al*.[325].

- *Risk of bias in individual studies (item 12)*: The quality of all studies included in this systematic review, was investigated using the NOS[245]. This was not intended to exclude any studies but rather to identify potential pitfalls that needed to be considered. Importantly, all studies obtained at least a rating of "moderate" (NOS 6) score. The four studies obtaining the lowest score resulted from problems with the comparability of cases and controls[202,299,304] or an insufficient description of the diagnostics of cases[314]. Despite this, all included studies were of a sufficiently high quality to be included in the analysis.

- *Synthesis of results (item 14)*: As specified by the PRISMA statement, the outcomes of the included studies in a systematic review can be presented and compared in a meta-analysis using statistical tests and forest plots[243]. The idea was considered although the highly heterogeneous methodologies of the included studies precluded an effective analysis. Thus, it was decided not to include a meta-analysis as part of study II. Instead, the methodologies and outcomes of the included studies were summarized and presented in tables.

- *Risk of bias across studies (item 15)*: To identify potentially missed articles, the references of the included articles were searched which did not identify any additional studies. Only studies in English or Danish were considered eligible for inclusion, which may have further introduced a risk of missing studies. Amongst the included studies, especially for ADHD, there were both studies reporting differences in gut microbiota and studies only finding minor to no variations in gut microbiota. This suggests that selective publishing might not have been a significant element in publication bias in this study, although it cannot be ruled out.

- *Results and discussion (items 17*-26): Results were described using tables to better provide an overview. Described results were assessed based on whether an overall difference was observed for alpha- or beta-diversity. After this, overlapping differences in specific bacteria were discussed, although the large methodological variations made this challenging.

Overall, this systematic review followed the items specified by the PRISMA statement[243] except for elements related to statistical analysis and meta-analyses. It would theoretically be possible to have performed subgroup analysis by extracting the available sequencing data for a combined analysis or to compare the demographics of the individual groups. However, the heterogeneous methodologies would have made this challenging. Publication bias was assessed to some extent but could not be completely ruled out.

4.2.3 STRENGTHS AND LIMITATIONS

Limitations

Some limitations need to be addressed, most of which are intrinsic to systematic reviews. First, as mentioned, we cannot rule out the risk of publication bias. A common issue within research is that it is easier to publish data showing differences compared to studies showing no effects[243]. It might have been beneficial to also have included a systematic search of published protocols. However, no protocols were

identified in the systematic search and none of the included studies referred to a published protocol, implying that this is not currently practiced within the field of gut microbiota in neurodevelopmental disorder. A related bias is the outcome reporting bias, describing the tendency to only report outcomes with a result[326]. Indeed, several studies were included that did not state whether they observed variations in alpha-diversity as evident in table 2. Finally, new studies where MESH had not been adequately assigned at the time of the systematic search might have been missed in this systematic review. A subsequent systematic search (June 2021) did not, however, reveal non-included studies before the original deadline for the search in the systematic review. Finally, the highly heterogeneous methodologies of the included studies precluded the planned use of a meta-analysis.

Despite these limitations, this systematic review provided a good and robust overview of the state of the research in the gut microbiota in individuals with ADHD and ASD at the time of 22nd of July 2019 and uncovered several leads for future studies. Furthermore, to our knowledge, this is the first study that compares the gut microbiota in individuals with ADHD and ASD, which is important due to the clinical and genetic overlap between the disorders[19,21].

4.3 STUDY III: CHILDREN WITH ATTENTION-DEFICIT HYPERACTIVITY DISORDER OR AUTISM SPECTRUM DISORDER SHARE DISTINCT MICROBIOTA COMPOSITIONS AND GASTROINTESTINAL PERMEABILITY

Aim of study III:

To investigate whether individuals with ADHD and/or ASD shared gut microbiota variations and to describe the gut microbiota associated with these disorders. A secondary aim was to investigate whether gut microbiota in individuals with ADHD and/or ASD were associated with changes in intestinal permeability.

4.3.1 MAIN FINDINGS

As evident from study II, the gut microbiota of children with ADHD or ASD has not been investigated in parallel, despite having several clinical[16,17], symptomological[19], and genetic[21] overlaps. Furthermore, the highly heterogenous methodologies preclude comparison of the gut microbiotas observed in previous studies investigating ADHD or ASD separately. Therefore, in study III we set out to investigate whether the disorders shared gut microbiota signatures.

In this study, 95 children or adolescents were recruited, who were diagnosed with either ADHD (n=32), ASD (n=12), or comorbid ADHD/ASD (n=11). As controls, 40 children and adolescents without neurodevelopmental disorders were recruited, consisting of siblings of individuals with ADHD (n= 14), ASD (n=5), and comorbid ADHD/ASD (n=4), as well as non-related children (n=17). The gut microbiota in these study participants was investigated using Illumina sequencing of the V4 hypervariable region of the 16S rRNA gene, leading to measures of microbial alpha- and beta-diversity, as well as measures on differentially abundant bacterial compositions. All the fecal samples were collected based on the experience obtained from study I. To evaluate the gut permeability in the included children, the calprotectin concentrations in all fecal samples, as well as the LBP concentration in a total of 85 blood plasma samples were measured using immunoassays.

While we did not observe any differences in alpha-diversity between any of the investigated diagnostic groups, the bacterial beta-diversity of children with ADHD or ASD was significantly different from non-related controls for both weighted and unweighted UniFrac (adjusted $p<0.05$ for all, unpublished data). This is consistent with previous studies indicating that the gut microbiota of individuals with ADHD or ASD separately are associated with differences in gut microbiota[170,293,295,299,300,315]. No significant differences were observed in beta-diversity between cases and their non-affected siblings, which suggests that environmental factors may explain some of the differences between cases and non-related controls. Intriguingly, the beta-diversity of both ADHD, ASD, and comorbid ADHD/ASD were highly similar using both Bray-Curtis dissimilarity (adjusted p=0.639), weighted UniFrac (adjusted p=0.645), and unweighted UniFrac (adjusted p=0.377).

Compositional analyses (unpublished data) further revealed that children with ADHD as well as children with ASD shared an increased relative abundance of the genus *Streptococcus,* as well as a decreased relative abundance of the families *Muribaculaceae* and *Suterellaceae*, and the genera *Sutterella* and *Coprobacter*. It is unclear how these variations may impact the presentation of the neurodevelopment disorders and whether they represent functional changes in the gut microbiota. However, these observations suggest that similar mechanisms are behind gut microbiota variations in both disorders, and are consistent with the high degree of clinical, symptomatologic[18], and genetic overlap[327,328] between the disorders as previously mentioned. The observation of increased relative abundance of *Streptococcus* in both disorders is perplexing since several previous studies have reported a reduced relative abundance of this genus in individuals with ASD[294,304,305,312,313,329]. However, a study by Li *et al*.[306] matched our results. The cause of this discrepancy is unknown but may reflect differences in methodology, including targeting different hypervariable regions as well as the use of bead-beating to improve detection of gram-positive bacteria[210]. Interestingly, a previous study has reported that children diagnosed with ADHD often had a history of previous streptococcal

infections, thus supporting the involvement of *Streptococcus* in neurodevelopmental disorders[330].

Looking at the individual disorders separately (unpublished data), ADHD was observed to be associated with changes amongst several low-abundant genera, which is consistent with the previous observations[299,300]. For individuals with ASD, the bacterial variations were more pronounced, with an increased relative abundance of the bacterial families *Enterobacteriaceae*, *Peptostreptococcaeae*, *Lactobacillaceae,* as well as the genera *Eubacterium* and *Streptococcus*. While previous studies on gut microbiota in individuals with ASD have revealed inconsistencies, with bacterial compositions associated with ASD, overall the observation of marked bacterial variations matches earlier observations[305,307,314,316].
To investigate how the gut microbiota affected the body, we measured indicators of the leaky gut hypothesis (unpublished data). No significant differences were observed for fecal calprotectin. However, the blood plasma concentration of LBP was significantly higher in individuals with ADHD (adjusted p=0.038), ASD (adjusted p=0.005), and comorbid ADHD/ASD (adjusted p=0.039) compared to non-related controls, indicating increased gut permeability in children with these disorders[153]. Previous studies have similarly observed increased gut permeability in patients with ADHD or ASD as indicated by increased serum concentrations of zonulin in children with ADHD[191] and increased serum LPS in adults with ASD[192]. Increases in plasma LBP have previously been linked to inflammation[149] due to the strong immune-stimulating effects of LPS[163]. Future studies are needed to investigate if the increased plasma LBP observed in this study is related to the tendency for inflammation previously reported among children with ADHD[200] and ASD[202,203,331]. Furthermore, more studies are needed to elucidate the interaction between gut microbiota and gastrointestinal functioning.

In conclusion, this case-control study revealed that children with ADHD and ASD shared gut microbiota distinct from those of non-related children without these disorders. These variations were further associated with an increased blood plasma concentration of LBP, indicating an increased gut permeability. The implications of these observations require further studies to elucidate.

4.3.2 METHODOLOGICAL CONSIDERATIONS

In this section, the study design and choices of methods for study III are discussed.

Study participants aged 5-17 years were recruited to this study. This age group reflects a compromise between avoiding confounding factors and obtaining a sufficiently high study population. Childhood was preferred to more accurately reflect the association between gut microbiota and ADHD and ASD rather than just reflecting variations

introduced by lifestyle, age[88,332], or development of other conditions[138,333,334]. While it would be beneficial to avoid the effects of puberty, this would have risked reducing the available study population too much as indicated by Dalsgaard *et al*.[9] Importantly, while puberty might be expected to introduce changes in gut microbiota due to changes in hormonal status, Xuan *et al.*[335] recently showed that the effects of puberty on alpha-or beta-diversity were minor. Furthermore, we did not detect any significant age differences between any of the groups, indicating that participant age did not serve as a major confounder.

The selection of control groups can have major implications on the ability to detect variations in gut microbiota. Originally, we aimed to only include siblings as controls to reduce environmental and genetic confounders as previously identified in sibling studies[46,47]. However, due to the heritable nature of the disorders[46,47] and the highly diverse sets of symptoms associated with ADHD and ASD[4,5], sibling controls also introduced a risk of recruiting controls that later presented with ADHD or ASD. This was observed when one sibling to a child with ADHD was diagnosed with ADHD at the end of the study period. Based on these considerations, it was decided to also include a non-related control group. In future studies, non-related controls need to be considered from the beginning in parallel with sibling controls.

Several types of pharmacological interventions have been associated with gut microbiota variations[97], and thus, it was considered whether to exclude children treated with ADHD medication. However, making ADHD medication an exclusion criterion would risk only leaving children with milder cases of ADHD in the study. Alternatively, children should be asked to withhold medication during the study period, which was considered unethical. While Sukmajaya *et al*.[324] have reported that there were no clear effects of the common ADHD medication Methylphenidate on gut microbiota, this should still be considered a potential confounder in this study. Importantly, in the current study, we did not observe any drug-specific variation in gut microbiota.

As previously mentioned, the choice of a target region in 16S rRNA gene sequencing can influence which bacteria were identified during the study. As evident for study II, most previous studies utilized the V3-V4 region, whereas, in study III, we used the V4 region. This choice resulted from an increased drive towards standardization within microbiota studies. Since the V4 region has proven robust and has been implemented as the target region in the major global studies Earth Microbiome Project[259] and American Gut[72], this region is becoming more commonly used. It should be noted that the selection of the V4 target region makes comparisons of study III to previous studies more cumbersome[69,210].

While fecal samples are frequently used as markers of the gut microbiota, previous studies have reported marked differences between the fecal microbiota and the microbiota inhabiting the intestinal mucosa[68,69]. To optimally represent the bacterial

interactions with the gastrointestinal tract, a sample needs to be taken directly from the gastrointestinal lumen. This might, however, introduce ethical and recruitment problems.

Finally, 16S rRNA gene amplicon sequencing of fecal samples is less precise than shotgun sequencing. It is therefore important to remember that 16S rRNA gene amplicon sequencing of fecal samples serves as an approximation of the true gut microbiota[69].

4.3.3 STRENGTHS AND LIMITATIONS

Certain limitations need to be addressed for this study. A total of 95 study participants were recruited although originally, we had planned to include 100 children with ADHD, 50 with ASD, 50 with comorbid ADHD/ASD, and a total of 100 controls. Following the initial interview, 146 children were recruited to the project with 97 children managing to deliver at least one of the required samples (one child with ADHD and one with comorbid ADHD/ASD did not deliver fecal samples and were thus excluded from study III), resulting in a dropout rate following the initial inclusion of 33.6 %. The main reason given for both initial reluctance to the project, as well as for dropout were stress and lack of mental energy during everyday life for both children and parents. This is a common feature in families with one or more children with neurodevelopmental disorders[336]. The low sample size amongst children with ASD and comorbid ADHD/ASD potentially reduced the power of the study to be able to detect gut microbiota signatures specifically associated with these disorders. Despite this, significant differences could be obtained in this study, although low-scale variations for ASD and comorbid ADHD/ASD might have been obscured. Secondly, all case-control studies suffer from selection bias, where the study participants recruited do not adequately represent the total population[337,338]. Benedict *et al*.[337] have previously described that study participants recruited through social media tended to differ in education status, income, and ethnicity[337], while Russel *et al*.[338] reported that studies on ASD tend to have selection bias against children with intellectual disability. These observations are consistent with the experiences obtained during recruitment in this study where families were more likely to not be interested or to drop out if they faced emotional or economic issues based on their child's disorder. Finally, as previously mentioned, amplicon sequencing of the 16S rRNA gene cannot reliably be used for species-level identification[291] which limited us to genus and above. Using whole-genome sequencing might have enabled us to describe species-level variations in the gut microbiota of ADHD and/or ASD[211].

Despite these limitations, this study has several strengths as well. To the best of our knowledge, this is the first study that has compared the gut microbiota of ADHD and ASD in parallel, as well as including the comorbid diagnosis as a separate category.

This has allowed an understanding of the shared effects of the gut microbiota in the two disorders. Secondly, the study participants have been characterized using widely used diagnostic instruments, which assists with comparisons with other studies. The combination of calprotectin and LBP measures as indicators of increased intestinal permeability, allowed us to evaluate whether the increased permeability resulted from inflammation of the gastrointestinal tract as an increased fecal calprotectin would have indicated[265]. Finally, the use of ASVs as opposed to OTUs allowed a greater resolution in this study compared to several previous studies[261].

CHAPTER 5. CONCLUSIONS

In this section, the conclusions for the individual studies are presented followed by an overall conclusion for the Ph.D. thesis.

In the methodological study I, we found that overall, both storage at -20 °C in a home freezer, as well as the use of the storage buffers PSP buffer, DNA/RNA shield, and RNA*later*, could effectively maintain the donor gut microbiota signature. While the use of storage buffers could introduce minor effects within the individual donors, storage at -20 °C was similar to immediate storage of fecal samples at -80 °C. The experiences from study I formed the basis for producing a strategy for sample collection in study III.

In the systematic review, study II, we demonstrated that despite a high degree of methodological heterogenicity, overall studies agreed that cases with ADHD or ASD possess a gut microbiota different from that of non-affected controls. While differences in methodology precluded the identification of an ADHD-or ASD-specific microbial signature, in individuals with ASD, several bacterial variations were indicated. These included an increased relative abundance of *Bacteroides* and a decreased relative abundance of *Streptococcus*. For individuals with ADHD, no consistently varying bacterial genera were identified. As a result, gut microbiota in individuals with ADHD and ASD could not be reliably compared.

The experiences from study II made it clear that gut microbiota in individuals with ADHD and ASD needed to be investigated in parallel and stressed the importance of standardization of methodology. This directly leads to the objectives of study III. Here we demonstrated for the first time that children with ADHD and ASD share a gut microbiota distinct from non-related controls. These variations were mostly observed for beta-diversity and were characterized by an increased relative abundance of the genus *Streptococcus* and a decreased relative abundance of the genera *Sutterella* and *Coprobacter*. Furthermore, children with ADHD and ASD possessed a higher plasma LBP concentration, suggesting that they have increased gastrointestinal permeability. Future studies are needed to unravel the implications of these variations for the behavioral and clinical characteristics of the disorders.

Overall, the aims of this Ph.D. project were fulfilled as we were able to show that children with ADHD and/or ASD do indeed possess a gut microbiota profile distinct from non-affected controls. However, the issue of heterogeneous results is still present. Both studies II and III identified *Streptococcus* as an important bacterium in distinguishing neurodevelopmental disorders from controls, however, they did not

agree on the direction of change. The most important discovery of this study was the shared gut microbiota between children with ADHD and ASD.

CHAPTER 6.
FUTURE PERSPECTIVES

While the observation of shared gut microbiota between individuals with ADHD and ASD is intriguing, the low sample size for ASD and comorbid ADHD/ASD means that the observation should be confirmed in larger studies. These studies would benefit from using whole-genome sequencing to enable the detection of variations at the species level[291]. This would also remove the bias introduced through the selection of different amplicon sequencing targets that contributed to parts of the methodological heterogeneity in study II.

Based on the findings presented in this Ph.D. thesis, it is evident that there is an association between gut microbiota and the neurodevelopmental disorders ADHD and ASD. However, more studies are urgently needed to elucidate the nature of this association, as wells as its clinical potential. This requires a better understanding of how gut microbiota interacts with the body in children with these disorders. Here, the observation of increased plasma LBP in children with ADHD, ASD, and comorbid ADHD/ASD observed in study III is of great interest due to its strong immune-stimulating effect[149]. Previous studies have reported an increased inflammatory state in ADHD[199,200] and ASD[170,202,293], and thus future studies should examine the correlation between gut microbiota, intestinal permeability, and inflammation, as well as how this correlates with symptoms of ADHD and ASD and the common somatic symptoms observed in these disorders. We are currently finishing analyses of the immune involvement in the group of participants included in study III, which will be compared to the measures of LBP and microbiota. Another potential mediator of gut microbiota function is through the production of psychoactive metabolites[339]. Variations in bacterial genes involved in the metabolic pathways of serotonin[340] and dopamine[299,340] have been reported in ADHD, with Aarts *et al*.[299] reporting that this variation was linked to alterations in reward anticipation. Similarly, several metabolic pathways have been demonstrated to be altered in the gut microbiota of children with ASD[190,202,304,305,308,312]. Based on the observed similarities in gut microbiota between ADHD and ASD, future studies should investigate whether the bacterial metabolites are also shared, and how these affect the body of children with the disorders.

All studies within the effects of gut microbiota suffer from the issue of the chicken or the egg: do gut microbiota variations cause ADHD and ASD or do common features involved in ADHD and ASD results in the development of a different gut microbiota environment? The common gastrointestinal symptoms in children with ADHD[165] or ASD[341], the observation of increased plasma concentrations of LBP in children with ADHD and/or ASD in study III, as well as previous reports of increased blood LPS in children with ASD[163,192] and serum zonulin for ADHD[191] point to variations in the

gastrointestinal tract in children with these disorders. Thus, studies are needed that investigated the condition of the gastrointestinal tract in children with these disorders.

As mentioned in the background section, Kang *et al*.[181] recently performed a clinical trial where they demonstrated that fecal microbiota transplant from non-affected individuals into children with ASD, introduced significant and long-term improvements in behavioral symptoms and gastrointestinal problems[181,182]. These observations combined with our observation of shared gut microbiota in children with ADHD and ASD warrant larger randomized controlled trials in the future for these diagnoses.

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PAPERS

PAPER I

Interpersonal Variations in Gut Microbiota Profiles Supersedes the Effects of Differing Fecal Storage Conditions

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Interpersonal Variations in Gut Microbiota Profiles Supersedes the Effects of Differing Fecal Storage Conditions

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Due to ease of acquisition, fecal samples are often used in studies investigating gut microbiota. Improper handling of these samples can lead to bacterial growth and alter bacterial composition. While freezing samples at −80 °C is considered gold standard, this is not suitable for studies utilizing self-sampling by lay participants or field studies. Thus to effectively prevent bacterial growth, techniques that allow efficient fecal storage outside laboratory facilities are needed. Fecal samples were collected from three donors. From each donor feces, 45 samples were collected and stored either freshly frozen at −80 or −20 °C, or in three separate storage buffers at room temperature or 4 °C for 24 or 72 hours. Bacterial composition was analyzed using Illumina amplicon sequencing of the V4 region of the 16 S rRNA gene. While storage conditions did affect bacterial composition and diversity compared to storage at −80 °C, the variation between donors superseded the variations introduced by storage. Samples stored at −20 °C most closely resembled those stored at −80 °C. When investigating variations in bacterial composition between separate study populations, fecal samples can efficiently be stored in −20 °C freezers or in one of the presented storage buffers, without severe alterations in bacterial composition.

Careful handling of biological samples is essential to avoid introduction of bias into the results. This is especially true for bacteria that may continue to grow during storage, changing the bacterial composition of samples[1–3]. Maintenance of bacterial composition is essential for studies investigating the role of gut bacteria in disease development. These gut bacteria, along with viruses, archaea and fungi, are collectively known as the gut microbiota, and has been implicated in maintenance of health[4]. Dysbiosis of the gut microbiota has conversely been associated with several disorders like infectious and autoimmune diseases[5–7], obesity[8,9], affective and neurodevelopmental disorders[10–12].

Bacterial composition in feces is regularly used as a representative for investigation of gut microbiota, and is mapped through sequencing of the 16 S ribosomal RNA (rRNA) gene[13,14]. Several bacterial species, including *Faecalibacterium*, normally found in the human gastrointestinal system, are anaerobic bacteria whose growth is severely limited when exposed to air. In contrast, the growth of aerobic bacteria may be enhanced following defecation, and the subsequent altered bacterial composition may introduce severe bias to gut microbiota studies[1]. To prevent bacterial growth following collection, the gold standard consists of immediate freezing of feces at −80 °C or in liquid nitrogen[2,15,16]. This procedure may not always be readily available since many studies rely on self-sampling by the involved study participants, where fecal samples are stored in domestic freezers[2,17]. These samples may be exposed to temperature fluctuations due to automatic defrost cycles, frost accumulations and partial thawing during transport to the research fascilities[17]. Still other studies rely on sample collection in areas without access to proper freezing facilities[14]. One way to overcome these challenges is to use storage buffers or reagents that may aid in stabilizing the fecal samples at room temperature. However, for these to function as proper replacements for freezing, it is crucial that the bacterial composition is not compromised or altered during the handling period. Good results have been obtained using a number of storage conditions, including −20 °C[18–22], OMNIgene®•GUT[16,17,23], Fecal Occult Blood Test cards[15,24], FTA cards®[17,21] or >95% but not < 95% ethanol[17,21,22]. Several studies have similarly investigated the commonly available RNAlater® buffer, but with

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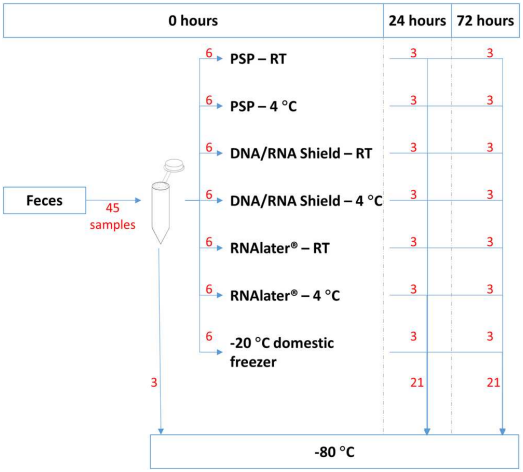

Figure 1. Overview of storage conditions for each of the three separate donor feces. 45 fecal samples were obtained from each donor feces. The samples were processed in triplicates and either stored directly at −80 °C, at −20 °C for 24 or 72 hours, or in one of the following buffers: DNA/RNA Shield, PSP Buffer, or RNAlater at 4 °C/RT, prior to freezing at −80 °C. Red numbers indicate number of samples that progress to this storage methodology.

mixed to negative results on the ability to sustain a microbiota profile[15–19,21,22,25]. Not all of the storage methods are, however, easily usable for lay participants which may introduce sampling variations, while other like ethanol can introduce transport restrictions[21]. PSP buffer is marked as a collection buffer that can be directly applied to the subsequent DNA extraction protocol. A study by Wu *et al.*[26] showed that Stool DNA Stabilizer from the PSP® Spin Stool DNA Plus Kit (PSP buffer) was able to sustain gut microbiota profile effectively for up to 48 hours. Another study investigated the effects of DNA/RNA shield™, and found that the buffer was effective at maintaining microbiota profiles and DNA quality in sheep[27]. While positive results have been obtained with DNA/RNA shield and PSP buffer, they have not adequately been compared to other methodologies usable by lay participants, like domestic freezers. Here we aimed to investigate differing storage methodologies suitable for self-sampling by lay participants, and their effects on bacterial compositions in fecal samples.

Results

This study investigated how storage of fecal samples in DNA/RNA shield, RNAlater, PSP buffer or frozen at −20 °C affected the resulting DNA output and bacterial composition compared to −80 °C. A total of 135 fecal samples were investigated, originating from three different donors, as illustrated in Fig. 1. DNA output was evaluated using yield, purity, and integrity of the extracted DNA. Effects on bacterial composition and diversity were investigated by 16 S rRNA gene sequencing. Following quality filtering and chimera removal, 2,906,405 16 S rRNA sequence reads were obtained, corresponding to a mean number of 21,529 ± 4,594 reads per sample. The mean number of reads per donor were 18,763 ± 2,878, 20,857 ± 3,721, and 24,967 ± 4667 for donor A, B, and C respectively. A total of 1,060 unique Operational Taxonomic Units (OTUs) were identified, with 95.5% being identified on the phylum taxonomic level, 80.1% on family level, 54.0% on genus level and 0.1% on species level. Importantly, all samples produced higher reads and OTU counts compared to negative controls, and comparable or higher number of reads and OTU compared to the anaerobic based positive control sample. A rarefaction curve was produced showing a good sequencing coverage (See Supplementary Images Section 1.1).

DNA levels increase in two of three storage buffers compared to freezing, probably due to bacterial growth. To investigate the effects of fecal storage methodologies on bacterial growth, we measured the quantity of DNA following extraction. DNA yields were comparable for samples stored at −80 °C, −20 °C, and in DNA/RNA Shield. However, a higher DNA yield was observed for samples stored in PSP buffer or RNAlater ($p < 0.05$, Fig. 2), indicating that these buffers may lead to increased bacterial growth. Neither temperature nor storage time affected DNA yield significantly.

DNA integrity and purity is not affected by use of storage buffers. For proper downstream analyses of bacterial DNA, it is important that DNA has good purity and is relatively intact. The purity of extracted DNA was measured using the 260/280 nm absorbance ratio (Fig. 3a). While variations did occur, all storage conditions maintained DNA with an $A_{260/280}$ OD ratio localized between 1.8 and 2.0. To ensure that DNA was not degraded during storage of DNA extraction, DNA integrity was investigated using agarose gel electrophoresis. For all samples, DNA was visible as a smear with the strongest signal observed between 10,000 and 20,000 bp (Fig. 3b). No visible differences in DNA fragment size were observed between storage conditions or durations.

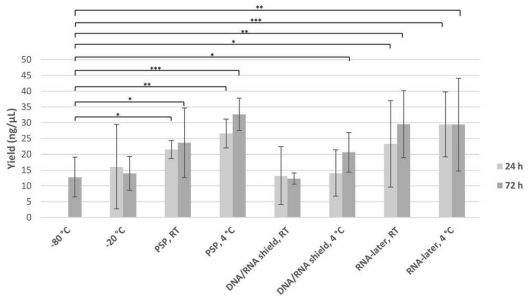

Figure 2. DNA yield. Yield of DNA from each storage condition of fecal samples, as measured using Qubit™ Fluorometric Quantification.

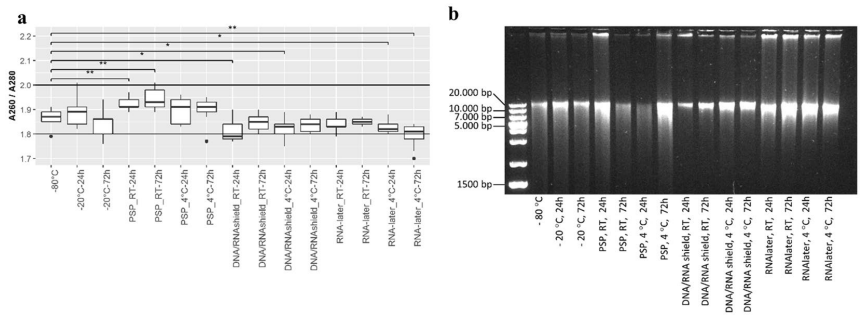

Figure 3. Integrity and purity of DNA extracted from each storage condition. (**a**) Purity of DNA extracted from fecal samples from each storage condition as measured by spectrophotometry. *$p < 0.05$. (**b**) Representative 1% agarose gel showing the effect of the different storage conditions on the integrity of DNA extracted from stool from donor A. Comparable results were observed for all replicates and donors from donor A, B and C. All agarose gels can be observed in Supplementary Images Section 1.3. RT: Room temperature.

Interpersonal differences in bacterial composition are larger than differences introduced through differing fecal storage conditions. Storage of fecal samples outside −80 °C may favor growth of individual bacterial genera following delivery that may skew bacterial composition. We therefore evaluated the effects of storage conditions on bacterial composition and diversity, using a Principal Component Analysis (PCA) of Hellinger Distance between OTU abundances (Fig. 4)[28]. We found that β diversity clearly superseded variations introduced by differing storage conditions in all donors. We next investigated factors influencing α diversity of samples, using bacterial richness, diversity and variability of all samples within donors. No effects of storage were found in bacterial richness ($p < 0.05$, Fig. 5a), whereas a non-significant ($p < 0.05$) altered Shannon diversity index was observed in samples stored in buffers compared to samples stored at −80 °C, most noticeable in samples from donor B and C (Fig. 5b). Within individual donors, samples stored at −20 °C and −80 °C clustered together and were combined. Samples stored using RNAlater, DNA/RNA shield, or PSP buffer, clustered based on buffer type, separate from frozen samples. (Fig. 6b,d,f). To investigate which bacteria were affected by differing storage conditions, the 25 most abundant genera for each donor were identified (Fig. 6a,c,e). For all donors *Faecalibacterium* was more abundant while *Alistipes* was less abundant in storage buffers compared to samples stored at −80 °C and −20 °C. This was especially evident in samples stored in PSP buffer in donor A and B. Interestingly, for donor C, an OTU from the phylum *Cyanobacteria* was observed to constitute a large percentage of the total OTU abundance in all samples stored in storage buffers but not for samples frozen at −20 °C or −80 °C. Importantly, for all samples, the variations introduced by storage methodology were larger than differences between individual triplicates (see Supplementary Images Section 1.2).

Discussion

Proper handling of fecal samples prior to DNA extraction is imperative for studies on the effects of gut microbiota on health, as biases can easily be introduced depending on choice of sampling and storage methodology[2]. In this study, we investigated the effects of different storage conditions on composition of bacterial communities in fecal samples from three healthy donors using Illumina sequencing of the V4 region of the 16 S rRNA gene.

For all samples, the variations in bacterial composition between individual donors, clearly superseded variations introduced by storage condition or duration. This is in line with other studies which reported that the

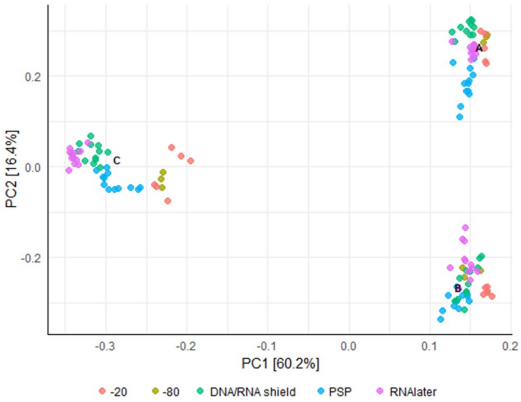

Figure 4. Differences in bacterial composition between storage conditions and the individual donors A, B and C. Clustering based on donor identity was seen using PCA plot using Hellinger Distance. Minor storage specific clustering was observed, but this was superseded by interdonor variation. PC1 explains 60.1% of variability between samples whereas PC2 explains 16.5%. All samples were frozen at −80 °C for a minimum of 24 hours following end of storage. PC: Principal component.

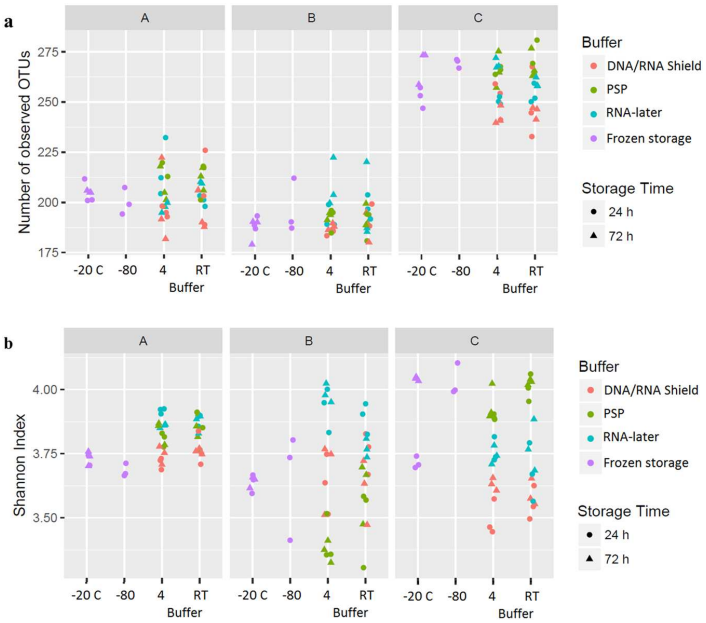

Figure 5. Bacterial α-diversity in feces from different study participants stored under differing conditions. The bacterial diversity was evaluated based on (**a**) number of different OTUs observed in each sample, and (**b**) the Shannon Diversity Index.

bacterial signature of donor identity was maintained despite differing storage methodologies[2,20,22]. This substantial β diversity is most likely caused by differences in donor specific factors including diet, lifestyle[29,30] and genetics[31,32], which may hide more subtle variations in bacterial composition. To better determine the effects of differing storage methodologies, bacterial composition and diversity was assessed separately for each donor. We found, that storage conditions, but not durations affected α diversity of samples. The bacterial composition of fecal samples stored at −20 °C was highly correlated with that of feces stored at −80 °C, confirming previous reports that storage at −20 °C is effective for studies investigating microbiota of feces[17,21,22]. All storage buffers

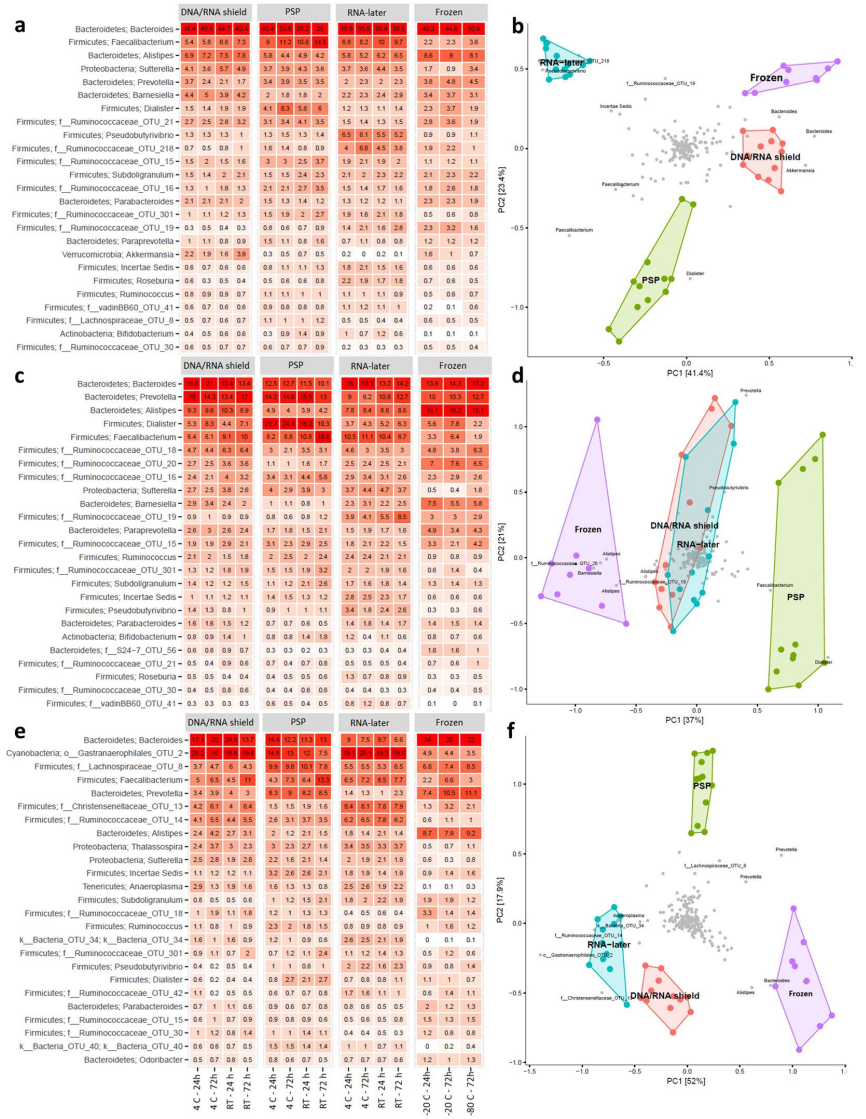

Figure 6. Bacterial composition of differing study participants and storage methodologies. Differences in bacterial compositions between individual storage conditions in study participant A (**a**,**b**), B (**c**,**d**) and C (**e**,**f**). Image (**a**,**c**,**e**) consists of heat maps representing the 25 most common genera/OTUs in different fecal storage conditions in the individual donor. Numbers describe the percentage of total reads that consists of this specific OTU. Each name consists of phylum name followed by a more specific genus name. If no genus name was available, the best assignment is shown. Image (**b**,**d**,**f**) visualize PCA plots. Within individual donors, storage methodology dependent clustering was observed using Hellinger Distance. Samples stored at −20 °C and −80 °C clustered together and were therefore combined. PC1 covers 41.1%, 37% and 52% of variation while PC2 represent 23.4%, 21% and 17.9% of variation respectively. Frozen: samples stored at either −80 °C or −20 °C, PC: Principal component; RT: Room temperature.

had minor variations in α diversity compared to samples stored at −80 °C, but no clear superior or inferior buffer could be recognized. To determine the cause of differing α diversities and DNA yields in buffers compared to frozen samples, we looked at composition of individual bacterial genera. Especially the bacterial genus

Faecalibacterium was found to be more abundant in fecal samples stored in buffers compared to feces stored at −80 °C. This bacterial genus has previously been shown to be insufficiently stabilized by other buffers[16], and thus we suggest that studies investigating these bacteria should be conducted with care. While the use of storage buffers did result in minor variations in α diversity, all storage conditions were capable of maintaining the inter-individual variations.

During DNA extraction, we found that it was difficult to fully separate RNAlater and feces using centrifugation, an observation that has also previously been reported[27,33]. Since RNAlater can influence subsequent cell lysis during DNA extraction[33], this could affect the microbiota profile. In two out of three participants, we observed a slightly reduced composition of bacteria belonging to the *Bacteroidetes* phylum and especially the genus *Prevotella* in samples stored in RNAlater, similar to observations by Hale *et al.*[21] and Sinha *et al.*[24] but in contrast to results obtained from Vogtmann *et al.*[34]. While RNAlater was suitable for distinguishing participant identity after storage, it was not optimal for storage of feces for microbiota studies. PSP was found to increase the proportion of bacterial genera belonging to the phylum *Firmicutes*. A similar observation was observed by Wu *et al.*[26], who suggested that PSP enables increased recovery of *Firmicutes*. The implications of this observation are not clear and need to be investigated in future studies. Finally, DNA/RNA shield had overall a bacterial composition close to that of frozen samples. This is not comparable to the results by Menke *et al.*[27] who found a significantly lower diversity of bacteria in sheep fecal samples stored in DNA/RNA shield compared to frozen samples. This discrepancy may however, be due to different core bacterial microbiotas between human and sheep. Overall, while each buffer possess limitations, all variations caused by buffers were superseded by participant identity.

Bead beating is normally utilized in microbiota studies, to enable extraction of DNA from bacteria that are difficult to lyse, like Gram-positive bacteria[25,35]. Excessive bead beating may however, result in DNA shearing and a reduced recovery of vulnerable bacteria[35,36]. We therefore analyzed the integrity of the extracted DNA. The majority had a length located between 10,000 and 20,000 bp, which is in agreement with previous reports of bacterial DNA sizes from freshly extracted feces[25,36]. Overall, all storage conditions produced a high DNA output with good purity and integrity, suitable for Illumina sequencing of the 16 S rRNA gene.

This study has certain limitations. First, the microbiota composition throughout an entire stool sample has been shown to be heterogeneous, which may introduce bias[18], and effective homogenization of feces prior to sample collection has been recommended[25]. However, this study aimed to investigate techniques feasible for lay participants, where homogenization is seldom possible[18]. Despite the lack of homogenization, our study did not find separate clustering of triplicates based on location but rather based on donor first, followed by storage methodology. Secondly, only three study participants were included, which limits the power of this study. The aim of this study was, however, to compare how different storage conditions affected the bacterial composition of fecal samples. For this purpose, testing the same storage conditions of several replicate samples from the same original feces (45 samples from each participant for a total of 135 fecal samples), was judged to more effectively assess the effects of storage compared to few samples from several participants. Using several samples from several participants would however, increase the power of this study. Finally, we did not compare the different storage conditions to a freshly extracted sample. This was precluded since conditions compatible with home sampling was the main scope of our study. Importantly, however, a previous study by Fouhy *et al.*[37], found that storage of fecal samples at −80 °C maintained a microbiota composition similar to freshly extracted samples.

Despite these limitations, this study has a number of strengths. We employed a structured study design with comparison of a high number of different storage conditions. Importantly, the methods are of low cost and fully suitable for home collection. These results are therefore very relevant for large-scale microbiota studies. Additionally, in order to secure the validity of our results and avoid misinterpretation, we included triplicate experiments, which resulted in a large sample size. Finally, all sequencings have been compared to an anaerobic digester that covers several differing bacterial phyla and genera, thus ensuring a good sequencing coverage.

Conclusions

While previous studies have investigated the effects of storage on the bacterial composition of fecal samples, the current study provides an in-depth investigation of the effects of storage methodologies suitable for home sampling by study participants compared to the gold standards of laboratory facilities.

Differing storage methodologies did introduce minor variations in bacterial composition, but the β diversity clearly superseded the variations introduced by storage. Thus, all investigated storage methodologies are suitable for stabilizing fecal samples for microbiota studies, with feces stored at −20 °C most closely resembling feces stored at −80 °C.

Methods

Sample collection and storage conditions. Fresh feces was collected from three healthy anonymous donors. As each storage condition was tested in triplicates, 45 samples (200 mg ± 50 mg of feces per sample) per donor was analyzed, resulting in a total of 135 fecal samples being analyzed for this study (Fig. 1). Briefly, three fecal samples were immediately frozen at −80 °C, while another three samples were frozen at −20 °C for 24 or 72 hours in a domestic freezer with manual defrost(EUC19001W, Electrolux). The remaining samples were stored in either DNA/RNA Shield™ (Zymo Research, USA), Stool DNA Stabilizer from the PSP® Spin Stool DNA Plus Kit (PSP buffer, Stratec Molecular, Germany), or RNAlater® (Invitrogen, Thermo Fisher Scientific, USA) at room temperature or 4 °C, for either 24 or 72 hours. Subsequently, all samples were collectively stored at −80 °C to rule out bias due to differences in low temperature exposure.

DNA extraction. Bacterial DNA was isolated from fecal samples using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN®, Germany) automated on a QIAcube® (QIAGEN), according to manufacturer's protocol with the

addition of a manual pretreatment step to enhance lysis of gram positive bacteria. In this pretreatment step, fecal samples containing storage buffer were centrifuged for 5 min at 14,500 x g, the supernatant was discarded, and all samples, including frozen samples, were resuspended in InhibitEX® lysis buffer. Bead beating was performed using a single 5 mm stainless steel ball (QIAGEN) on a TissueLyser LT (QIAGEN) for 4 min at 30 Hz. This was followed by lysis at 95 °C for 5 min, and 200 μL of the resulting lysate solution was transferred to spin columns continuing with the standard protocol.

Purity of the extracted DNA was evaluated spectrophotometrically, with a Nanodrop™ Lite (Thermo Fisher Scientific) using the $A_{260/280}$ OD ratio. The concentration of DNA was measured using the Qubit™ HS Assay (Thermo Fisher Scientific). Finally, DNA integrity was evaluated by agarose gel electrophoresis.

16S rRNA gene sequencing. Bacterial 16S rRNA amplicon sequencing targeting the V4 variable region, was performed by DNAsense ApS (Denmark), and followed a modified version of an Illumina protocol[38]. An initial amplicon PCR and clean-up was performed as described by Albertsen *et al.*[35], using the V4 primers (5′-GTGCCAGCMGCCGCGGTAA and 5′-GGACTACHVGGGTWTCTAAT[39]), and 35 cycles of amplification. This was followed by an index PCR and clean-up[38]. Finally, samples were pooled and sequenced on a MiSeq™ (Illumina®, USA) as previously described[40], but with the addition of 20% PhiX control library (Illumina, USA) to measure error rate during sequencing, a negative control (nuclease-free water) to eliminate background, and a positive control (complex sample obtained from an anaerobic digester system) to ensure efficient sequencing.

Statistics and data analysis. Quality of sequencing reads were analyzed using FastQC (Babraham Bioinformatics, UK). Forward reads were quality trimmed using Trimmomatic v 0.32[41] utilizing settings SLIDINGWINDOW:5:3 and MINLEN:250 to remove reads with a Phred Score below 20 and discard reads shorter than 250 bp. The reads were next dereplicated and formatted for use in the UPARSE workflow[42]. The first 250 bp of all reads were clustered using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTUs were clustered based on 97% identity and chimeras removed using the usearch v. 7.0.1090 –usearch_global command with –id 0.97. Taxonomy was assigned using the RDP classifier[43] as implemented in the parallel_assign_taxonomy_RDP.py script in QIIME[44] using the MiDAS database v. 1.20[45].

Data analysis was performed in R[46] through the Rstudio IDE (http://www.rstudio.com/) using the ampvis2 package v.2.3.11[35], as well as Microsoft Office Excel 2013. α diversity was determined using OTU richness and Shannon Diversity Index as implemented in the amp_alphadiv command of the ampvis2 packet in R. β diversity was determined using PCA clustering and heat maps in the ampvis2 package. PCA plot using Hellinger Distance was performed using the amp_ordinate function, while heatmaps displaying the composition of the 25 most common OTUs were generated using the amp_heatmap function. For continuous data like $A_{260/280}$ OD ratio, DNA concentration, OTU richness, and Shannon Diversity Index, distribution was tested using Shapiro-Wilks test while variance was tested using Bartlett's test. Normal distributed data was expressed by mean values and analyzed using ANOVA followed by Tukeys post-hoc test, while data that was not normal distributed or did not have equal variances, was expressed as median values and analyzed using Kruskal-Wallis Test followed by Dunn's post hoc test. Differences were considered statistical significant for $p < 0.05$.

Ethical approval. The study protocol was reviewed by the Regional Ethical Committee of Northern Denmark. Since no personal information were collected from study participants and no intervention was performed, the Ethical Committee judged that no further approval was required.

The study was carried out in accordance to the guidelines provided by the Ethical Committee of Northern Denmark concerning anonymized biological material.

Availability of Data

Sample information (Supplementary_metadata) and OTU-tables (Supplementary_Otutable) generated during sequencing and used for bioinformatics are available at the Dryad Digital Repository: doi:10.5061/dryad.61r43kd.

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Author Contributions

S.E.S. and C.B.N. designed the study; C.B.N. performed experiments; S.E.S. supervised the project; S.E.S., S.O.H. and C.B.N. all participated in data analysis; S.E.S. and C.B.N. prepared the manuscript, while S.E.S., S.O.H. and C.B.N. contributed to finalizing the manuscript. All authors read and approved the final manuscript.

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PAPER II

Gut microbiota profiles of autism spectrum disorder and attention deficit/hyperactivity disorder: A systematic literature review.

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REVIEW

Gut microbiota profiles of autism spectrum disorder and attention deficit/hyperactivity disorder: A systematic literature review.

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ABSTRACT

Accumulating evidence has implicated an involvement of the gut-brain axis in autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD), however with highly diverse results. This systematic review aims to describe and evaluate studies investigating the gut microbiota composition in individuals with ASD or ADHD and to evaluate if variations in gut microbiota are associated with these disorders.

Twenty-four articles were identified in a systematic literature search of PubMed and Embase up to July 22, 2019. They consisted of 20 studies investigating ASD and four studies investigating ADHD. For ASD, several studies agreed on an overall difference in β-diversity, although no consistent bacterial variation between all studies was reported. For ADHD, the results were more diverse, with no clear differences observed.

Several common characteristics in gut microbiota function were identified for ASD compared to controls. In contrast, highly heterogeneous results were reported for ADHD, and thus the association between gut microbiota composition and ADHD remains unclear. For both disorders, methodological differences hampered the comparison of studies.

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Autism Spectrum Disorder; attention-deficit hyperactivity disorder; neurodevelopmental disorders; microbiota; microbiome; systematic Review; gut-Brain axis

Introduction

In recent years, the prevalence of autism spectrum disorder (ASD) and attention-deficit disorder/attention-deficit/hyperactivity disorder (in this paper both disorders are referred as ADHD) has increased. Globally, ASD and ADHD are estimated to affect 1.0–2.0%[1,2] and 7.2%,[3] respectively, of all children and both disorders are associated with potentially severe social, adaptive, and educational problems. Thus, the development of these disorders is receiving increasing research attention.[4,5] While ASD describes a range of abnormalities characterized by impairment of social and communicative skills combined with restrictive-repetitive behavior,[6] ADHD is defined by symptoms of inattention, impulsivity, and/or hyperactivity.[7] Despite these seemingly different symptoms, the two disorders are often co-existing, with previous studies reporting that up to 63% of ASD cases displayed ADHD symptoms.[7] Both disorders have substantial genetic contributions, with heritability estimates of approximately 54% and 74% for ASD and ADHD, respectively.[8,9] Furthermore, the two disorders share several genetic variants.[10] Despite these clear genetic involvements, heritability has not been able to satisfyingly predict the disorders, and instead, they are believed to be the result of a complex interaction between genetic and environmental factors.[8,9,11]

For both ASD and ADHD, gastrointestinal (GI) symptoms are common, with constipation, diarrhea, and GI pain affecting up to 70% of ASD patients,[12,13] and the intensity of GI symptoms are positively correlated with ASD severity.[12,14] Similar to ASD, GI symptoms like constipation, fecal incontinence, and abdominal pain are commonly reported by ADHD patients.[15,16] Based on these observations, dietary interventions have been attempted. These include the use of gluten and casein-free diets for management of ASD symptoms,[17] and, for ADHD, omega-3, and −6 fatty acid supplementation and removal of food coloring. Results have varied, although reduction of core

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symptoms of ASD and ADHD has been demonstrated.[17,18] Overall, while dietary interventions have not been successful in treating ASD or ADHD, the symptom improvements observed may suggest that components of the GI tract are involved in ASD or ADHD. A number of studies has suggested that the gut microbiota may serve as one of these components.[19–21]

The GI tract contains a thriving population of bacteria, that together with viruses, fungi, protozoa, and archaea, forms a community of microorganisms termed the gut microbiota.[22] Variations within the normal bacterial composition have been associated with the development of different pathophysiological conditions including type 2 diabetes,[23] obesity,[24] and inflammatory disorders.[25–27] Studies have indicated that GI bacteria are involved in a bidirectional interaction with the brain, which has been shown to be important for normal neurodevelopment.[28,29] Disruption of this interaction, termed the "gut-brain axis", has been hypothesized to be implicated in several neurological or psychiatric disorders like Parkinson's disease,[30] depression,[31,32] or bipolar disorder.[31] A number of direct and indirect contributing pathophysiological mechanisms has been proposed by which the gut microbiota may impact these disorders. Direct mechanisms include stimulation of the vagus nerve[34,35] and production of psychoactive metabolites as reported for ASD.[36] The indirect mechanisms include a number of functional differences that may result in increased GI tract permeability,[37] allowing leakage of bacterial products like lipopolysaccharides to the blood, and thus result in low-grade systemic inflammation.[30,38]

Several studies have examined the role of gut microbiota in ASD using culturing or targeted approaches.[19,39,40] A recent preclinical study demonstrated that autism-like behavior could be transferred to mice through fecal microbiota transplant from children with ASD.[41] Other studies have attempted probiotic treatment, but with conflicting results.[42–44] Although gut microbiota has been suggested as a potential clinical target in treatment, the role of gut microbiota in ASD is still not completely understood.[45] Unlike ASD, information on the role of gut microbiota in ADHD is limited. In the few studies published so far, a lowered abundance of fecal *Bifidobacterium* in infancy or early life infection with *Streptococcus* has been associated with increased risk of developing ADHD.[20,46]

Despite several indications suggesting a relationship between an altered gut microbiota and ASD or ADHD, the nature of this involvement is still not clear. In order to facilitate the use of gut microbiota in improving diagnosis and treatment of core symptomology in ASD and ADHD, we require a better understanding of which bacteria are associated with these neurodevelopmental disorders, and how they affect their pathophysiological characteristics.

Thus, the aim of this study was to investigate and describe the current findings relating to altered gut microbiota composition in individuals with ASD and ADHD.

Methods

Search protocol

The protocol for this systematic review was registered at the International Prospective Register of Systematic Reviews (PROSPERO) under the ID number CRD42018111458, prior to commencement of this study. The guidelines provided by the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA) were used.[47] A systematic search strategy was performed prior to July 22, 2019, using the databases PubMed and Embase, with no restrictions on publication year. Search strings were tailored for each database, based on existing publications, and are visualized in Table 1. The references of included studies were screened to identify potentially missed studies.

Eligibility criteria

Articles were included based on the following criteria: The included studies must be original studies performed in humans, diagnosed with one or both of the following diagnoses: ASD (299.00 or 299.80 according to the "Diagnostic and Statistical Manual of Mental Disorders" (DSM)-IV or 5 criteria and F84.0, F84.1, F84.5, or F84.8 according to the "International Statistical Classification of Diseases and Related Health Problems" (ICD)-10 criteria) or ADHD (314.00 or 314.01 according to DSM-IV or 5 or F90.0, F90.1 or F98.8 according to the ICD-10 criteria). The complete microbial community must be assessed in fecal samples. The microbial community should be compared to

Table 1. Search terms used for the systematic search. Use of "AND" or "OR" in the search engines has been indicated.

	Horizontal lines divided by "AND"	
	Cases	Outcome
Search terms (Vertical lines divided by "OR")	• Neurodevelopmental disorders[MESH] • Attention Deficit Disorder*[Text Word] • Attention Deficit Hyperactivity Disorder[Text Word] • ADHD[Text Word] • ADD[Text Word] • Autism[text word] • "Autism Spectrum Disorder"[MESH] • Neurodevelopmental*[text word] • Neurodevelopmental disorder[MESH]	• (Microbiology[MESH] OR Microbiology[Subheading] OR Microbiology[Text Word])AND(Feces"[MESH] OR Gastrointestinal Tract[MESH]) • Gastrointestinal Microbiome[MESH] • Gastrointestinal Microbiome*[text word] • Gastrointestinal Microbiot*[text word] • Gut microbiot*[text word] • Gut microbiome*[text word] • Intestinal Microbiot*[text word] • Intestinal Microbiome*[text word].

a control group without either ASD or ADHD. The articles must be written in English or Danish.

Articles were excluded if they included less than 10 study participants or focused on co-morbidity between ASD/ADHD and other disorders.

All inclusion or exclusion criteria are available at the Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies (CAMARADES) website SyRF (http://app.syrf.org.uk/projects/5ffc6aab-3415-43b3-be6a-1fc5084f08fa/detail).

Study selection

Articles obtained from the literature searches were combined, and duplicates were removed using the automatic function implemented in the reference manager Mendeley (https://www.mendeley.com/). The articles were analyzed in two stages: Initially, titles and abstracts were screened independently by two researchers (CBN and JKK), using SyRF (http://syrf.org.uk/), according to the eligibility criteria. Next, the included articles were subjected to whole-paper revision. Disagreements between the two reviewers were resolved by consensus-based discussion, and, if necessary, a third reviewer was involved (SES).

Data extraction and quality assessment

Data were extracted to a Microsoft Excel file (Supplementary Table 1), focusing on demographics, diagnostic methodology, microbiota assessment methodology, bacterial richness, diversity, and taxonomic bacterial composition (phylum, family, genus, and species only). Meta-analysis was not performed due to the heterogeneity of methodology.

Quality assessment of the included studies was evaluated, using the Newcastle-Ottawa Scale (NOS) for case–control studies.[48] NOS contains three criteria: selection (are cases and controls effective community controls?), comparability (are cases and controls comparable?), and exposure (how are diagnosis and microbiota assessed?). A quality score ranging from 0 to 10 was obtained by the use of a rating algorithm previously described:[45] 0–5 (poor), 6–7 (moderate), and 8–10 (high).

Results

Study selection

The initial database search generated 1,841 articles, which were reduced to 1,532 unique articles after automatic duplicate removal. Subsequent screening of titles and abstracts resulted in 62 articles assigned to whole paper revision. During whole paper revision, 38 articles were excluded due to non-complete eligibility criteria upon closer inspection. This included articles that only investigated a subset of the gut microbiota (n = 12); were conference abstracts (n = 12); characterized gut microbiota in GI biopsies or urine samples rather than fecal samples (n = 5); only characterized gut microbiota following pro- or prebiotic intervention (n = 4); were duplicates of already included studies (n = 2); had less than 10 study participants (n = 2); or did not compare the gut microbiota to a control population (n = 1). Finally, 24 original articles were included in this systematic review. These articles included 20 articles investigating ASD[49–68] and 4 investigating ADHD[69–72] (Figure 1, supplementary data 1). None

of the studies included study participants with both disorders. [Figure 1 near here]

As indicated in Table 2, all studies received a NOS score ranging from six (moderate) to eight (high). Four studies,[51,56,58,61] all investigating ASD, received a score of eight (high), due to matching cases and controls on other variables than age alone. Conversely, four studies (three investigating ASD and one investigating ADHD)[53,55,68,69] received a score of six (moderate), due to inadequate description of samples,[68] controls represented by children undergoing surgery and thus not being representative community controls,[53] or controls being older than ASD or ADHD cases (tables 3 and 4).[55,69] The remaining studies all received a score of seven (moderate).

Characteristics of studies investigating ASD or ADHD

Demographics of the included studies are seen in tables 3 and 4 for ASD and ADHD, respectively. Geographically, the studies were performed in USA,[49,51,62,63,65,67,68] Europe,[53,55,60,61,64,69,70] Taiwan,[72] People's Republic of China,[52,54,56-59,71] Australia,[50] and India.[66] The studies investigating ASD included in total 733 cases and 590 controls (138 siblings and 452 non-related controls), whereas the studies of ADHD included in total 114 cases and 156 controls (21 siblings and 135 non-related controls).

The majority of studies used non-related participants as controls, while four studies compared ASD cases to siblings,[50,61,63,66] and two (one ASD and one ADHD) compared cases to both siblings and non-related controls.[49,69] It is noteworthy, that while the majority of studies included cases and controls younger than 18 y only, one study investigating ADHD[69] included cases and controls older than 18 y. For most studies investigating ASD, there was a higher percentage of males among cases compared to controls (total of 74.6% for cases, 41.3% for siblings, and 63.3% for non-related controls, for all studies providing this). This was also true for studies

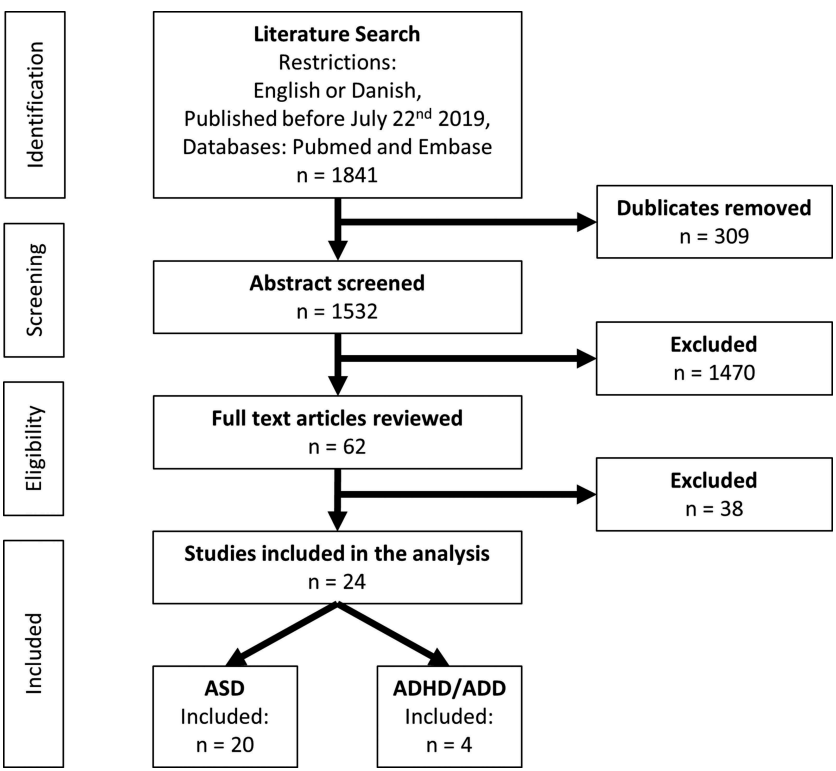

Figure 1. PRISMA flow diagram, summarizing the studies identified during the systematic literature search and reviewing process.

Table 2. Quality assessment of included studies based on the newcastle-ottawa scale for case–control studies. The articles were rated based on selection and characterization of cases and controls (Selection, max score 4), comparability between case and controls (Comparability, max score 2), and ascertainment of effects of microbiota (Exposure, max score 4), for a potential score ranging from 0 to 10 points.

Study	Year	Selection	Comparability	Exposure	Total
ASD					
Finegold et al.[49]	2010	4	1	2	**7**
Gondalia et al.[50]	2012	4	1	2	**7**
De Angelis et al.[61]	2013	4	2	2	**8**
Kang et al.[62]	2013	4	1	2	**7**
Son et al.[63]	2015	4	1	2	**7**
Strati et al.[64]	2017	4	1	2	**7**
Kang et al.[65]	2017	4	1	2	**7**
Pulikkan et al.[66]	2018	4	1	2	**7**
Kang et al.[67]	2018	4	1	2	**7**
Berding et al.[68]	2018	3	1	2	**6**
Rose et al.[51]	2018	4	2	2	**8**
Zhang et al.[52]	2018	4	1	2	**7**
Coretti et al.[53]	2018	3	1	2	**6**
Li et al.[54]	2019	4	1	2	**7**
Carissimi et al.[55]	2019	4	0	2	**6**
Liu et al.[56]	2019	4	2	2	**8**
Zhai et al.[57]	2019	4	1	2	**7**
Ma et al.[58]	2019	4	2	2	**8**
Wang et al.[59]	2019	4	1	2	**7**
Plaza-Díaz et al.[60]	2019	3	2	2	**7**
ADHD					
Aarts et al.[69]	2017	4	0	2	**6**
Prehn-Kristensen et al.[70]	2018	4	1	2	**7**
Jiang et al.[71]	2018	4	1	2	**7**
Wang et al.[72]	2019	4	1	2	**7**

investigating ADHD (total of 77.2% for cases compared to 62.8% for controls). Only three studies used gender-matched cases and controls.[56,38,70] We also recorded information regarding the use of special diets or nutritional supplements, presence of GI symptoms as well as the use of medication. We found that for three studies investigating ASD[49,62,63] and one study investigating ADHD,[72] the diet, or use of probiotics, of cases differed from that of controls. Similarly, presence of GI symptoms (primarily constipation, but also diarrhea and abdominal pain) was common for ASD (reported in 31.5% of cases versus and 7.3% for controls), which was not seen for ADHD. All studies excluded participants who recently received antibiotics, while two studies investigating ADHD[69,70] included cases that received ADHD medication. No other medical treatments were observed to be prevalent in the studies (tables 3 and 4).

Handling and analysis of samples

A number of differences in sample handling and analyzes was observed between the individual studies, and are described in Table 5. Following sample collection, the majority of studies stored the fecal samples at either −20°C or −80°C, while other studies used preserving buffers[51,54,61,63] or stored samples at 4°C.[58,69,70] Two studies did not provide information on storage of samples.[55,62] For DNA extraction, most studies used commercial spin column-based extraction kits, with approximately half of the studies implementing pretreatment steps to increase DNA extraction from gram-positive bacteria.[51,54,57,60–65,67,70,72] All studies, with the exception of two that used metagenomic sequencing,[55,59] assessed fecal microbiota using amplicon sequencing of the 16 S ribosomal ribonucleic acid (rRNA) gene, targeting a number of hypervariable regions. Taxonomy was assessed using a variety of different databases, with Greengenes[51–54,64–68] being the most common.

Children and adolescents with ASD have distinct gut microbiota

The gut microbial communities of ASD cases were compared to controls, assessing α- and β-diversity as well as changes in individual bacterial abundances.

Table 3. Demographics of ASD cases included in this systematic review. No studies included participants that received antibiotic treatment. In the row labeled "Total", the total number of participants, and total gender distribution and GI symptoms percentage (for studies providing numbers) for all studies combined, are displayed.

		ASD						
Study	Country	Sample size		Gender (Male %)	Age (years)	Diagnostic instrument	GI symptoms (% of total)	Special diet
Finegold et al.[49]	USA	ASD:	33	72.7	2-13	N/A	100.0	Diet: Unspecified number of cases used special diet.
		SIB:	7	28.6			0.0	
		Ctrl:	8	62.5			0.0	
Gondalia et al.[50]	Australia	ASD:	51	82.4	2-12	N/A	54.9	N/A
		SIB:	53	35.8			7.5	
De Angelis et al.[61]	Italy	ASD:	20	46.7	4-10	ADI-R, ADOS	0.0	No special diet
		SIB:	10				0.0	
Kang et al.[62]	USA	ASD:	20	90.0	3-16	ADI-R, ADOS, ATEC, PDD-BI	100.0	Diet: 5 cases. Dietary supplements: 13 cases + 8 Ctrls.
		Ctrl:	20	85.0			0.0	
Son et al.[63]	USA	ASD:	59	88.1	7-14	N/A	42.4	Diet: 4 cases + 1 Ctrl.
		SIB:	44	47.7		(DSM-IV)	29.5	
Strati et al.[64]	USA	ASD:	40	77.5	Mean age: 11.1	N/A	12.5	No special diet
		Ctrl:	40	70.0		(DSM-IV)	27.5	
Kang et al.[65]	Italy	ASD:	18	88.9	7-16	ADI-R	100.0	No special diet
		Ctrl:	20	90.0			0.0	
Pulikkan et al.[66]	India	ASD:	30	93.3	3-16	CARS, ISAA	Common for ASD cases	No special diet
		SIB:	24	62.5				
Kang et al.[67]	USA	ASD:	2123	65.2	4-17	ATEC, PDD-BI	Common for ASD cases	N/A
		Ctrl:		95.6				
Berding et al.[68]	USA	ASD:	26	73.1	2-7	N/A	Common for ASD cases	No special diet
		Ctrl:	32	59.4				
Rose et al.[51]	USA	ASD:	50	84.0	<13	ADI-R, ADOS	42.0	No special diet
		Ctrl:	41	92.7			17.1	
Zhang et al.[52]	People's Republic of China	ASD:	35	82.9	3-8	N/A	31.4–60.0	No special diet
		Ctrl:	6	83.3		(DSM-IV)	0.0	
Coretti et al.[53]	Italy	ASD:	11	81.8	2-4	ADOS2, ADI-R, GMDS, VABS, CARS	18.2	No special diet
		Ctrl:	14	57.1			0.0	
Li et al.[54]	People's Republic of China	ASD:	59	84.7	2-10	ADOS, ABC	50.8	No special diet
		Ctrl:	30	66.7			23.3	
Carissimi et al.[55]	Italy	ASD:	16	100.0	2-6	GMDS, ADOS2	Common for ASD cases	N/A
		Ctrl:	7	28.6	5-16			
Liu et al.[56]	People's Republic of China	ASD:	30	83.3	2.5-18	N/A	30.0	No special diet
		Ctrl:	20	80.0		(DSM-5, ICD-10)	5.0	
Zhai et al.[57]	People's Republic of China	ASD:	78	71.8	Mean age: 4.9	ATEC	Common for ASD cases	N/A
		Ctrl:	58	53.4				
Ma et al.[58]	People's Republic of China	ASD:	45	86.7	6-9	CARS	N/A	No dietary differences between cases and controls
		Ctrl:	45	86.7				
Wang et al.[59]	People's Republic of China	ASD:	43	83.7	2-8	N/A	44.2	No difference between cases and controls
		Ctrl:	31	58.1		(DSM-5)	0.0	
Plaza-Díaz et al.[60]	Spain	ASD:	48	Matched	2-6	ADI-R, ADOS, PDD-BI	Common for ASD cases	N/A
		Ctrl:	57					
Total		**ASD**	**733**	**74.6**	**2-18**		**31.5**	
		SIB	**138**	**41.3**			**12.3**	
		Ctrl	**452**	**63.3**			**5.8**	

The most consistent microbiota differences are visualized in Table 6, with a more comprehensive list presented in supplementary data 1. Highly heterogeneous results were obtained for α- (number of species and their diversity within samples) and β-diversity. (difference in bacterial composition between

Table 4. Demographics of ADHD cases included in this systematic review. No studies included participants that received antibiotic treatment. In the row labeled "Total", the total number of participants, and total gender distribution and GI symptoms percentage (for studies providing numbers) for all studies combined, are displayed.

Study	Country	Sample size	Gender (Male %)	Age (years)	Diagnostic instrument	GI symptoms (% of total)	Special diet or ADHD medication
Aarts et al.[69]	The Netherlands	ADHD: 19 SIB: 21 Ctrl: 56	68.4 SIB/ctrl: 53.2	Mean age: ADHD: 19.5 SIB+Ctrl: 27.1	K-SADS-PL	N/A	Diet: N/A Unspecified number of cases received ADHD medication
Prehn-Kristensen et al.[70]	Germany	ADHD: 14 Ctrl: 17	100.0 100.0	Mean age: 11.9	K-SADS-PL	N/A	Diet: No difference in diet. 10 cases received Methylphenidate.
Jiang et al.[71]	People's Republic of China	ADHD: 51 Ctrl: 32	74.5 68.8	6-10	K-SADS-PL	0.0 0.0	No special diet No pharmacological treatment of ADHD
Wang et al.[72]	Taiwan	ADHD: 30 Ctrl: 30	76.7 60.0	6-16	K-SADS-PL ADHD-RS	0.0 0.0	Diet of cases differed from that of controls. No pharmacological treatment of ADHD
Total		**ADHD 114** **SIB 21** **Ctrl 135**	**77.2** **SIB/ctrl 62.8**	**6-N/A**		**N/A/ 0.0**	

samples). The majority of studies did, however, find that the overall microbiota composition of ASD cases differed from that of controls.[49,51–54,56–58,61,64,67,68]

A number of differences was observed between ASD cases and controls when comparing the relative abundance of individual bacterial phyla and genera. For phyla, three studies reported increased relative abundance of *Proteobacteria* in ASD cases.[49,53,60] Nine studies reported altered *Firmicutes-Bacteroidetes* ratio, although they differed in the direction of change.[49,52,53,56,57,61,64,66,68] For bacterial genera, several studies reported increased relative abundance of *Bacteroides*,[49,53,57,61] *Barnesiella*,[56,61,67] *Clostridium*,[54,59,61,68] and *Roseburia*,[53,61,68] as well as reduced relative abundance of *Bifidobacterium*,[49,53,61,65,68] *Coprococcus*,[61,62,67] *Dialister*,[49,64,68] *Faecalibacterium*,[61,67,68] *Prevotella*,[54,61,62,67] and *Streptococcus*[49,52,53,56,61] in cases. However, no specific bacteria consistently differed between ASD cases or controls in all of the included studies. A few of the studies also looked at the effects of microbial differences; The metabolism was reported to be affected by several of the microbial changes associated with ASD.[52,53,55,56,58,59,61,68] This was especially true for short-chain fatty acids (SCFAs) metabolism, that was reported to be affected by changes in *Faecalibacterium, Ruminococcus*, and *Bifidobacterium* composition.[52,53,56,61,68] Further more, two studies reported that the gut microbiota of ASD was associated with increased concentrations of pro-inflammatory cytokines,[51,55] while Wang et al. reported that the increased *Clostridium* and *Bacteroides* associated with ASD resulted in reduced cortisol concentrations.

Studies investigating gut microbiota in ADHD cases yield inconclusive results

As for ASD, studies investigating the gut microbiota of ADHD compared to controls used α- and β-diversity as well as changes in individual bacterial abundances (Table 6, more comprehensive list in supplementary data 1). No clear overall conclusion could be drawn from the studies. The two studies originating from Europe observed that the gut microbiota β-diversity of ADHD cases differed from controls,[69,70] whereas none of the two East-Asian studies observed any significant differences.[71,72] Furthermore, changes in individual bacteria were inconsistent between the four studies. All found ADHD specific changes, but no studies agreed on what bacterial taxa differed. Three studies discussed the causes and effects of the gut microbial variations. Wang et al. reported increased *Bacteroides* in children with ADHD, which was correlated to dietary differences. Jiang et al. reported that *Faecalibacterium* was negatively associated with ADHD symptoms, while Aarts et al.[69] reported that genes encoding cyclohexadienyl dehydratase (CDT) had increased functionality in the ADHD-associated bacteria. The authors

Table 5. Handling of samples from cases and controls. N/A: No information provided.

Study	Sample storage	DNA extraction	Sequencing technique/target	Reference database
ASD				
Finegold et al.[49]	Transported overnight on ice	QIAamp DNA Stool mini kit	454 FLX pyrosequencing, 16 S rRNA	Custom database similar to RDP-II
Gondalia et al.[50]	Transported overnight on ice	QIAamp DNA Stool mini kit	454 FLX pyrosequencing, 16 S rRNA V1-V3 region	BLASTn
De Angelis et al.[61]	RNAlater, frozen at −80°C	Bead-beating. FastDNA pro soil-direct kit	454 FLX pyrosequencing, 16 S rRNA V1-V3 region	GenBank
Kang et al.[62]	−20°C for up to 24 hours	Bead-beating. QIAamp DNA Stool mini kit	454 FLX pyrosequencing, 16 S rRNA V2-V3 region	SSURef
Son et al.[63]	RNAlater, stored cold overnight	Bead-beating. ZR Fecal DNA MiniPre	Illumina sequencing, 16 S rRNA V1-V2 + V1-V3 region	SILVA
Strati et al.[64]	−80°C	Bead-beating. FastDNA Spin kit for feces	454 FLX pyrosequencing, 16 S rRNA V3-V5 region	Greengenes
Kang et al.[65]	N/A	Bead-beating. Powersoil DNA kit	Illumina Miseq, 16 S rRNA V4 region	Greengenes
Pulikkan et al.[66]	−80°C	QIAamp DNA Stool mini kit	Illumina sequencing, 16 S rRNA V3 region	Greengenes
Kang et al.[67]	−20°C for up to 24 hours	Bead-beating. Powersoil DNA kit	454 FLX pyrosequencing, 16 S rRNA V2-V3 region	Greengenes
Berding et al.[68]	−80°C	QIAamp Fast DNA Stool mini kit	Illumina sequencing, 16 S rRNA V2-V3 region	Greengenes
Rose et al.[51]	RNAlater, frozen at −20°C	Bead-beating. Powersoil DNA kit	Illumina sequencing, 16 S rRNA V3-V4 region	Greengenes
Zhang et al.[52]	−80°C within few hours	N/A	Illumina sequencing, 16 S rRNA V3-V4 region	Greengenes
Coretti et al.[53]	−80°C	QIAamp DNA Stool mini kit	Illumina Miseq, 16 S rRNA V3-V4 region	Greengenes
Li et al.[54]	99% ethanol. Later frozen at −80°C	Bead-beating. FastDNA Spin kit for feces	Illumina Hiseq, 16 S rRNA V1-V2 region	Greengenes
Carissimi et al.[55]	N/A	QIAamp DNA Stool mini kit	Illumina paired end Shotgun sequencing	-
Liu et al.[56]	−80°C within 30 min	QIAamp Fast DNA Stool mini kit	Illumina Miseq, 16 S rRNA V3-V4 region	SILVA
Zhai et al.[57]	Transported on ice	Bead-beating. FastDNA Spin kit for soil	Illumina Miseq, 16 S rRNA V3-V4 region	N/A
Ma et al.[58]	4°C for up to 12 hours	QIAamp Fast DNA Stool mini kit	Illumina Hiseq, 16 S rRNA V3-V4 region	SILVA
Wang et al.[59]	−80°C upon delivery at lab	StoolGen fecal DNA extraction kit	Illumina Hiseq Shotgun sequencing	-
Plaza-Díaz et al.[60]	−80°C upon delivery at lab	95°C pretreatment in lysis buffer. QIAamp DNA Stool mini kit	Illumina Miseq, 16 S rRNA V3-V4 region	RDP
ADHD				
Aarts et al.[69]	Stored at 4°C for up to 24 hours	Dneasy blood and tissue kit	454 FLX pyrosequencing, 16 S rRNA V3-V6 region	RDP
Prehn-Kristensen et al.[70]	Stored at 4°C	Bead-beating. FastDNA Spin kit for Soil	Illumina Miseq, 16 S rRNA V1-V2 region	N/A
Jiang et al.[71]	−20°C for up to 24 hours	QIAamp DNA Stool mini kit	Illumina Miseq, 16 S rRNA V3-V4 region	N/A
Wang et al.[72]	−20°C for up to 24 hours	Pretreatment with lysis buffer. QIAamp DNA Stool mini kit	Illumina Miseq, 16 S rRNA V3-V4 region	RDP

further reported that the increased abundance of CDT was significantly associated with decreased reward anticipation, previously reported in ADHD.[69]

Discussion

Understanding the microbial communities associated with ASD and ADHD has the potential of improving current treatment options for individuals with these disorders. While studies have attempted to utilize fecal microbiota transfer[65] or probiotics in the treatment of ASD or ADHD, results have been limited.[20,42,65] Given the large inter-individual variations in the healthy microbiome,[73] a better understanding of normal variation, as well as whether gut bacteria are involved in the etiology of ASD and

Table 6. Table depicting the most important observations on bacterial composition between ASD or ADHD cases, and controls. Only bacteria, for which two separate studies have agreed on the direction of difference, are displayed in the table. Empty boxes represent that no difference was reported for this measurement, between cases and controls in the represented study. For De Angelis et al.[57] autism and PDD-NOS were combined. ↑ = higher α diversity or bacteria are more abundant, in ASD/ADHD compared to control; ↓ = Lower α diversity or bacteria are less abundant, in ASD/ADHD compared to control; D = bacterial β-diversity differ between ASD or ADHD cases compared to controls. N = No difference in β-diversity. – = no information.

	ASD																				ADHD							
																			Total							Total	
Studies	49	50	61	62	63	64	65	66	67	68	51	52	53	54	55	56	57	58	59	60	↑	↓	69	70	71	72	↑	↓
α-diversity																												
Richness	↓		↑	↓					↓					↑			↑	↓	↓		3	5				↑	1	0
Diversity			↑	↓			↓		↓			↓	↑		↓	↓	↑	↓			3	7		↓		↑	1	1
β-diversity	D	N	D	N	N	D	N	N	D	D	D	D	D	D	-	D	D	D	-	-	D=12		D	D	N	N	D=2	
Phylum																												
Actinobacteria	↓												↓						↑	↑	2	2	↑				1	0
Bacteroidetes	↑		↑			↓		↓		↓		↑	↑				↑				5	3					0	0
Firmicutes	↓		↓			↑		↑		↑		↓				↓	↓				3	5					0	0
Proteobacteria	↑												↑							↑	3	0					0	0
Genera																												
Actinomyces												↓	↓								0	2					0	0
Bacillus																	↑			↑	2	0					0	0
Bacteroides	↑		↑										↑				↑				4	0		↑			1	0
Barnesiella			↑						↑							↑					3	0					0	0
Bifidobacterium	↓		↓				↓	↑		↓			↓							↑	2	5	↑				1	0
Butyricimonas										↓		↑								↑	2	1					0	0
Clostridium	↓		↑							↑				↑					↑		4	1					0	0
Collinsella	↓		↑			↑															2	1					0	0
Coprococcus			↓	↓					↓				↑								1	3					0	0
Dialister	↓					↓				↓											0	3			↓		0	1
Dorea			↑			↑															2	0					0	0
Enterococcus																↑				↑	2	0					0	0
Escherichia			↓									↓									0	2					0	0
Eubacterium			↓													↓					0	2					0	0
Faecalibacterium			↓						↓	↓			↑								1	3			↓		0	1
Lachnospira			↓										↑					↓			1	2					0	0
Lactobacillus						↑		↑													2	0				↓	0	1
Megaspaera								↑						↑							2	0					0	0
Oscillospira			↓										↑				↑				2	1					0	0
Parabacteroides	↑		↓			↓							↑				↑				3	2		↓			0	1
Prevotella			↓	↓					↓					↓						↑	1	4		↓			0	1
Pseudomonas														↑		↑					2	0					0	0
Roseburia			↑							↑			↑								3	0					0	0
Ruminococcus	↓		↑										↑								2	1					0	0
Streptococcus	↓		↓									↓	↓	↑		↓					1	5					0	0
Sutterella												↑					↑				2	0					0	0
Turicibacter	↓		↓																		0	2					0	0
Veillonella						↓						↓									0	2					0	0

ADHD, is needed to develop future microbiota-based treatments.

Gut microbiota of ASD and/or ADHD

In this systematic review, we sought to evaluate whether individuals with ASD or ADHD had a distinct microbiota composition compared to controls. Importantly, for ASD, the majority of studies identified that the gut microbiota of ASD cases differed from controls, although no specific bacteria was consistently altered across studies. As suggested by Turnbaugh et al.,[74] the microbiome of a pathologic condition can also be defined by an altered function rather than an altered bacterial composition. Amongst bacteria reported to have increased relative abundance in ASD cases, several genera has previously been associated with inflammation.[75–77] Conversely, several commensal bacteria with lower relative abundance are known to induce anti-inflammatory effects[78,79] or are involved in the maintenance of normal metabolism.[68,79–81] The findings have been supported by Rose et al.[51] and Carissimi et al.,[55] who reported that ASD cases had an increased concentration of pro-inflammatory cytokines. However, we still lack more in-depth analyzes in the functions affected by the gut microbiota in ASD. These include, but are not limited to, studies investigating bacterial metabolites and effects on inflammation and metabolism.

Compared to ASD, the number of published studies investigating the involvement of gut microbiota in ADHD are surprisingly limited. This is supported by a recent systematic review, where only two studies on ADHD and gut microbiota were identified; both studies are also included in the present review.[82] Amongst the included studies, the results were furthermore too heterogeneous to make confident conclusions regarding whether ADHD is associated

with a different gut microbiota profile. Reduced relative abundances were reported of the bacterial genera *Parabacteroides, Prevotella*,[70] *Faecalibacterium, Dialister*, and *Lactobacillus*[83] in ADHD cases compared to controls. These genera are known to assist with maintenance of the normal GI tract function,[78,81,84–86] which fits with the observed functional differences in carbohydrate and fat metabolism in ADHD, reported by Wang et al.[72] Both Aarts et al.[69] and Jiang et al.[71] reported a significant correlation between specific microbial differences and ADHD symptomology. While intriguing, more studies are urgently needed to further elucidate whether these microbial interactions might directly influence the pathophysiology of ADHD. A previous study by Cheng et al.[87] further reported that single nucleotide polymorphisms (SNPs) associated with the genus *Desulfovibrio* and the order *Clostridiales*, were enriched in ADHD cases, although we could not substantiate this observation. Interestingly, in a large study of microbiota–drug interactions, Zimmermann et al.[88] reported that certain gut bacteria could chemically modify the common ADHD drug Methylphenidate. Since the response of ADHD patients to medication differs,[89] studies are needed to investigate whether gut microbiota could be used to predict drug response in ADHD patients.

As previously mentioned, there is a high degree of overlap between ASD and ADHD. It is therefore interesting if the two disorders share gut microbiota variations. Both ASD and ADHD are associated with a lower relative abundance of commensal bacteria related to the maintenance of a healthy GI function, which may explain the high frequency of GI dysfunctional conditions. It is however important to note that the differences in methodologies and the reported heterogeneous microbiota compositions in the reviewed articles hamper our ability to investigate the possibility of a shared gut microbiota in ASD and ADHD.

Differences in methodology may explain the heterogeneous results

It is well known that several factors may have an influence on the composition of gut microbiota, including geographic, cultural, dietary, and demographical differences,[63,90–93] which may explain some of the observed discrepancies between different studies. Intriguingly, Winglee et al. showed that urbanized Chinese people had gut microbiota with closer resemblance to Americans rather than that of rural Chinese people.[94] This indicates that differences previously attributed to ethnical or geographical differences may instead be explained by differences in lifestyle. While a fiber-rich, plant-based diet is associated with a gut microbiota rich in the *Bacteroidetes* phylum and the genus *Prevotella*, a typical western diet is associated with increased *Firmicutes* and *Bacteroides*.[90,91,94,95] It is recognized, that children with ASD often have a lower vegetable intake compared to children without ASD, often due to selective eating and sensory disturbances,[96,97] and it is thus interesting, that several studies included in this systematic review reported increased *Bacteroides* and decreased *Prevotella* for ASD cases.[49,53,57,61,62,67]

GI dysfunctions, primarily constipation, and diarrhea, were common amongst the ASD cases in several of the studies included in this systematic review. As reported by Vandeputte et al.,[98] gut microbiota composition is highly associated with colon transit time as indicated by fecal consistency. While a fast transit time selects for fast-growing bacteria, the slow transit time observed in constipation enables more slowly growing bacteria to thrive. As a result, the increased presence of GI symptoms in ASD cases may explain some of the differences in gut microbiota observed between the studies.

Importantly, we observed that studies differed in selection of control groups. The majority of studies compared cases with non-related controls, some compared to siblings, and some to both groups, to correlate for similarities in environment. Finegold et al.[49] reported that the gut microbiota of siblings to children with ASD, had a bacterial composition resembling a middle group between ASD and non-related controls. This may explain why two studies using siblings as controls only observed none to minor bacterial differences compared to controls.[50,63]

While sequencing enables highly sensitive determination of the microbiota composition, several factors in conjunction with handling of samples may influence data output.[99,100] Among the studies included in this systematic review, several different storage techniques were utilized, ranging from lowered temperature to the use of storage buffers. While gut microbiota is robust, differences in storage can lead to growth or disruption

of susceptible bacteria, and thus result in differences in bacterial composition between studies.[99,101,102] Extraction of DNA from gram-positive bacteria is problematic, due to the presence of a thick cell wall, that can prevent effective bacterial lysis during DNA extraction.[100,103] This can lead to underrepresentation of gram-positive bacteria in studies investigating gut microbiota.[100] Only half the studies included in this systematic review took steps to increase DNA extraction from gram-positive bacteria. Despite this, we did not detect a clear pattern in differences in bacteria known to be difficult to extract, like the *Streptococcus* genera,[103] and the impact is thus uncertain. Finally, most of the included studies investigated microbiota composition by sequencing the different hypervariable regions of the 16 S rRNA gene. However, primers targeting different regions have different affinities to specific bacteria, and thus may capture different bacteria in the same samples.[100] This makes comparison of studies using primers targeting different regions problematic.

Besides differences in sample handling, the studies also differed in the choice of bioinformatics pipelines and reference databases. Two commonly used reference databases amongst the included studies were Greengenes (http://greengenes.secondgenome.com/) and SILVA (https://www.arb-silva.de/). As reported by Park et al.[104] these reference databases may not always identify the same microbial genera, which impairs proper comparison of studies. Here it is noteworthy that none of the included studies using the SILVA reference database identified differences in the *Bacteroidetes* phylum. In contrast, five out of nine studies using the Greengenes database reported differences for this phylum.

Overall, several methodological differences were observed between the studies included in this systematic review, but no single factor explained the heterogeneity. It is thus unclear whether the heterogeneous gut microbiota compositions for each disorder presented in this systematic review, represent natural variations, or whether several factors together cause these variations in gut microbiota.

Limitations

A number of limitations needs to be addressed: First, analysis of the included studies proved complicated, since they varied widely regarding methodology and demography. This made the performance of a meta-analysis unfeasible. Secondly, all systematic reviews are susceptible to publication bias, where studies reporting differences in microbiota composition between cases and controls are more likely to be published. We read the references of the included studies, to determine if other studies were missed in the systematic search. This did not reveal any additional studies, suggesting that we adequately covered the published literature. Finally, new studies may have been missed, if MESH terms had not been assigned at the time of the systematic search.

Conclusion

This systematic review has demonstrated that ASD and ADHD cases are associated with a gut microbiota different from controls without neurodevelopmental disorders. However, studies varied widely concerning methodology, resulting in highly heterogeneous gut microbiota compositions between studies. A specific ASD or ADHD-associated gut microbiota could therefore not be established, although, for ASD, a few shared functional differences were suggested. Future studies should consider investigating differences in gut microbiota function as well as composition. Furthermore, the differences in methodology and demography could have influenced the gut microbiota of the studies, and thus studies are needed that investigate the gut microbiota jointly in these often comorbid diagnoses.

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PAPER III

Children with Attention-Deficit Hyperactivity Disorder or Autism Spectrum Disorder Share Distinct Microbiota Compositions and gastrointestinal permeability

Caspar Bundgaard-Nielsen, Marlene B. Lauritsen, Julie K. Knudsen, Louise S. Rold, Peter Hindersson, Peter D. C. Leutscher, Søren Hagstrøm, Mette Nyegaard, and Suzette Sørensen

The paper is in preparation and has been made available for the Ph.D. committee.

Not available in the published version of the dissertation.

GUT AND NEURODEVELOPMENT: THE ROLE OF GUT MICROBIOTA DYSBIOSIS IN ATTENTION-DEFICIT HYPERACTIVITY DISORDER AND AUTISM SPECTRUM DISORDER.

APPENDIX

Ethical Approval

GUT AND NEURODEVELOPMENT: THE ROLE OF GUT MICROBIOTA DYSBIOSIS IN ATTENTION-DEFICIT HYPERACTIVITY DISORDER AND AUTISM SPECTRUM DISORDER.

REGION NORDJYLLAND
– i gode hænder

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Ref.: TH

Dato 19. september 2017

N-20170044
Gut and Neurodevelopment:
The Role of Gut Microbiota Dysbiosis in Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorder

Endelig godkendelse

Afgørelsen er truffet efter lov om videnskabsetisk behandling af sundhedsvidenskabelige forskningsprojekter (lov nr. 593 af 14. juni 2011).

Den Videnskabsetiske Komite for Region Nordjylland bekræfter modtagelsen af mail af 04-09-2017, som svar på komiteens afgørelse af 26-06-2017, hvori der opstilledes betingelser for godkendelsen af projektet.

Betingelserne for godkendelsen anses for opfyldt. Projektet er dermed endeligt godkendt.

Godkendelsen gælder til den **01-09-2020** og omfatter følgende dokumenter:

- Forsøgsprotokol, version **3**, af **04-09-2017**
- Deltagerinformation, version **3**, af **04-09-2017**
- Informeret samtykkeerklæring, version **3**, af **04-09-2017**

Godkendelsen gælder for de anmeldte forsøgssteder og den anmeldte forsøgsansvarlige i Danmark.

Iværksættelse af projektet i strid med godkendelsen kan straffes med bøde eller fængsel, jf. komitélovens § 41.

Ændringer:
Foretages der væsentlige ændringer i protokolmaterialet under gennemførelsen af projektet, skal disse anmeldes til komiteen i form af tillægsprotokoller. Ændringerne må først iværksættes efter godkendelse fra komiteen, jf. komitélovens § 27, stk. 1.

Anmeldelse af tillægsprotokoller skal ske elektronisk på www.dnvk.dk med det allerede tildelte anmeldelsesnummer og adgangskode.

Væsentlige ændringer er bl.a. ændringer, der kan få betydning for forsøgspersonernes sikkerhed, fortolkning af den videnskabelige dokumentation, som projektet bygger på samt gennemførelsen eller ledelsen af projektet. Det kan fx være ændringer i in- og eksklusionskriterier, forsøgsdesign, antal forsøgspersoner, forsøgsprocedurer, behandlingsvarighed, effektparametre, ændringer om de forsøgsansvarlige eller forsøgssteder samt indholdsmæssige ændringer i det skriftlige informationsmateriale til forsøgspersonerne.

Hvor nye oplysninger betyder, at forskeren overvejer at ændre proceduren eller stoppe forsøget, skal komiteen orienteres om det.

Bivirkninger og hændelser:
Løbende indberetning
Komiteen skal omgående underrettes, hvis der under projektet optræder formodet alvorlige, uventede bivirkninger eller alvorlige hændelser, jf. komitélovens § 30, stk. 1. Indberetningen skal ledsages af kommentarer om eventuelle konsekvenser for forsøget. Det er kun bivirkninger og hændelser forekommet i Danmark, der skal indberettes. Underretning skal ske senest 7 dage efter, at sponsor eller den forsøgsansvarlige har fået kendskab til tilfældet.

Ved indberetning kan anvendes et skema, der findes på www.dnvk.dk. Skemaet med bilag kan indsendes elektronisk ved anvendelse af digital signatur.

Årlig indberetning
Én gang årligt i hele forsøgsperioden skal komiteen have tilsendt en liste over alle formodet alvorlige (ventede og uventede) bivirkninger og alvorlige hændelser, som er indtruffet i forsøgsperioden sammen med en rapport om forsøgspersonernes sikkerhed, jf. komitélovens § 30, stk. 2.
Materialet skal være på dansk eller engelsk.

Ved indberetning skal anvendes et skema, der findes på www.dnvk.dk. Skemaet med bilag kan indsendes elektronisk ved anvendelse af digital signatur.

Afslutning:
Den forsøgsansvarlige skal senest 90 dage efter afslutningen af projektet underrette komiteen herom, jf. komitélovens § 31, stk. 1. Projektet regnes som afsluttet, når sidste forsøgsperson er afsluttet.

Ved indberetning kan anvendes et skema, der findes på www.dnvk.dk. Skemaet indsendes elektronisk ved anvendelse af digital signatur.

Afbrydes projektet tidligere end planlagt, skal en begrundelse herfor sendes til komiteen senest 15 dage efter, at beslutningen er truffet, jf. komitélovens § 31, stk. 2.

Hvis projektet ikke påbegyndes, skal dette samt årsagen hertil meddeles komiteen.

Komiteen beder om kopi af den afsluttende forskningsrapport eller publikation, jf. komitelovens § 28, stk. 2. Vi skal i den forbindelse gøre opmærksom på, at der er pligt til at offentliggøre både negative, positive og inkonklusive forsøgsresultater, jf. komitélovens § 20, stk. 1, nr. 8.

Tilsyn:
Komiteen fører tilsyn med, at projektet udføres i overensstemmelse med godkendelsen, jf. komitélovens §§ 28 og 29.

Med venlig hilsen

Tine Holland
Fuldmægtig

Kære Caspar Bundgaard-Nielsen

På vegne af Den Videnskabsetiske Komité for Region Nordjylland skal jeg hermed kvittere for modtagelse af orientering om ændring af godkendt (af komitéen) projektmateriale på baggrund af mindre ændringer i forhold til kontaktoplysninger i deltagerinformation samt mindre volume i forhold til den godkendte mængde blod, der udtages i forsøget.

Samtidig bekræftes det, at det fremadrettet vil være nedenstående dokumentversion, der er gældende og finder anvendelse i forsøget:

- Protokol, version **4**, af **4. september 2017**
- Skriftlig deltagerinformation, version **4**, indsendt af **15. december 2017**
- Informeret samtykkeerklæring, version **4**, indsendt af **15. december 2017**

Med venlig hilsen

Karina Schøler
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Gut and Neurodevelopment:
The Role of Gut Microbiota Dysbiosis in Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorder.

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Dato 8. marts 2019

Den Videnskabsetiske Komite for Region Nordjylland bekræfter modtagelsen af mails af 28. februar og 8. marts 2019, som svar på komiteens afgørelse af 20. februar 2019, hvori der opstilledes betingelser for godkendelsen af tillægsprotokol **1** til ovennævnte projekt.

Betingelserne for godkendelsen anses for opfyldt. Tillægsprotokollen er dermed **endeligt godkendt** som værende i overensstemmelse med bestemmelserne i lov nr. 593 af 14. juni 2011 (Komitéloven).

Godkendelsen omfatter:

- Forsøgsprotokol, version **5.0**, af **12.03.2019** indsendt af **28. februar 2019**
- Deltagerinformation, version **5**, indsendt af **8. marts 2019**
- Annoncetekst, version **1**, indsendt af **28. februar 2019**
- Presseopslag, version **1**, indsendt af **28. februar 2019**
- Tekst til webrekruttering af patienter, version **1**, indsendt af **28. februar 2019**
- Tekst til webrekruttering af kontroller, version **1**, indsendt af **28. februar 2019**
- Rekrutteringspjece, version **1**, af **12. februar 2019**

Komitéens godkendelse er gældende til den **1. september 2020.**

I medfør af Komitéloven har de videnskabsetiske komitéer til opgave at følge op på de godkendte projekter. I den forbindelse gør vi særligt opmærksom på følgende bestemmelser i loven om den forsøgsansvarliges forpligtelser:

§ 30. *Stk. 2.* Sponsor eller den forsøgsansvarlige skal én gang årligt i hele forsøgsperioden indsende en liste til den tilsynsførende komité over alle formodet alvorlige uventede bivirkninger, som er opstået i forsøgsperioden, og give oplysning om forsøgspersonernes sikkerhed. Medmindre forskningsprojektet angår kliniske forsøg med lægemidler omfattet af Lægemiddelstyrelsens tilsyn i medfør af lov om lægemidler, omfatter underretnings- og oplysningspligten endvidere alvorlige hændelser.

§ 31. Senest 90 dage efter afslutningen af et anmeldelsespligtigt sundhedsvidenskabeligt forskningsprojekt underretter den forsøgsansvarlige og sponsor i forening den tilsynsførende komité om, at projektet er afsluttet. *Stk. 2.* Afbrydes et forskningsprojekt, før det er planlagt afsluttet, underretter den forsøgsansvarlige og sponsor i forening den tilsynsførende komité om afbrydelsen, senest 15 dage efter at beslutningen herom blev truffet.

Afbrydelsen skal begrundes. Den tilsynsførende komité kan om fornødent afkræve den forsøgsansvarlige og sponsor en begrundet redegørelse.

Resultatet af projektet, eventuelt i form af en artikel, rapport eller lignende indsendes til komitéen, når det foreligger.

Alle henvendelser vedrørende projektet bedes rettet til komitéens sekretariat. Komitéens journalnummer **N-20170044** anført.

Med venlig hilsen

Karina Schøler
Specialkonsulent

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Dato 3. august 2020

Den Videnskabsetiske Komité for Region Nordjylland har den 3. august 2020 behandlet tillægsprotokol **2** til ovennævnte projekt.

Komitéen kunne godkende tillægsprotokollen som værende i overensstemmelse med bestemmelserne i lov nr. 593 af 14. juni 2011.

Godkendelsen omfatter:

- Forsøgsprotokol, version **6**, af **3. august 2020**
- Forlængelse af godkendelsesperioden

Komitéens godkendelse er gældende til den **31. august 2021.**

I medfør af Komitéloven har de videnskabsetiske komitéer til opgave at følge op på de godkendte projekter. I den forbindelse gør vi særligt opmærksom på følgende bestemmelser i loven om den forsøgsansvarliges forpligtelser:

§ 30. *Stk. 2.* Sponsor eller den forsøgsansvarlige skal én gang årligt i hele forsøgsperioden indsende en liste til den tilsynsførende komité over alle formodet alvorlige uventede bivirkninger, som er opstået i forsøgsperioden, og give oplysning om forsøgspersonernes sikkerhed. Medmindre forskningsprojektet angår kliniske forsøg med lægemidler omfattet af Lægemiddelstyrelsens tilsyn i medfør af lov om lægemidler, omfatter underretnings- og oplysningspligten endvidere alvorlige hændelser.

§ 31. Senest 90 dage efter afslutningen af et anmeldelsespligtigt sundhedsvidenskabeligt forskningsprojekt underretter den forsøgsansvarlige og sponsor i forening den tilsynsførende komité om, at projektet er afsluttet. *Stk. 2.* Afbrydes et forskningsprojekt, før det er planlagt afsluttet, underretter den forsøgsansvarlige og sponsor i forening den tilsynsførende komité om afbrydelsen, senest 15 dage efter at beslutningen herom blev truffet. Afbrydelsen skal begrundes. Den tilsynsførende komité kan om fornødent afkræve den forsøgsansvarlige og sponsor en begrundet redegørelse.

Resultatet af projektet, eventuelt i form af en artikel, rapport eller lignende indsendes til komitéen, når det foreligger.

Komitéen er ikke ressortmyndighed for regelsættet om databeskyttelse. Komitéen forudsætter, at projektet gennemføres i overensstemmelse med databeskyttelsesforordningen og databeskyttelsesloven.

Alle henvendelser vedrørende projektet bedes rettet til komitéens sekretariat. Komitéens journalnummer **N-20170044** bedes anført.

Med venlig hilsen

Ulla Bay Hansen
Sekretær

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