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Gut microbiome in adjustment disorder

Master's Thesis (30 ECTS)

Curriculum Bioengineering

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Gut microbiome in adjustment disorder

Abstract:

Adjustment disorder (AD) is a common psychiatric disorder with a general prevalence of <1% – 2%, yet research is limited. This study analyses whether the gut microbiome composition is associated with AD by comparing the gut microbiome composition between 236 participants with a history of AD diagnosis and 2270 controls. Alpha diversity using Shannon index was significantly higher in AD, but the association was not statistically significant after adjusting for covariates. Beta diversity analysis showed that the microbiome compositions between cases and controls are significantly different even after covariate adjustment. Differential abundance analysis found 133 bacteria, whose abundance was statistically different between the groups, but no associations were identified after correcting for multiple testing. In conclusion, there is suggestive evidence that the gut microbiome composition is associated with AD.

Keywords:

Gut microbiome, alpha diversity, beta diversity, differential abundance analysis

CERCS: Bioinformatics, medical informatics, biomathematics, biometrics

Soolestiku mikrobioomi roll kohanemishäire puhul

Lühikokkuvõte:

Kohanemishäire (adjustment disorder; AD) on levinud psühhiaatriline häire, mille levimus populatsioonid on 1-2%, kuid mida on vähe uuritud. Magistritöös uuritakse, kas soolestiku mikrobioom on seotud kohanemishäirega. Selleks analüüsitakse soolestiku mikrobioomi 236 indiviidi, kellel on minevikus tuvastatud kohanemishäire ning 2270 kontrolli, kellel häiret tuvastatud ei ole. Töös selgus, et mikrobioomi alfa-mitmekesisuse erinevus gruppide vahel osutus statistiliseks oluliseks, kuid seost ei tuvastatud pärast kovariaatidega kohandamist. Beeta- mitmekesisuse analüüs näitas, et soolestiku mikrobioomi kooslus on kohanemishäirega indiviididel kontrollidest statistiliselt erinev ka pärast kovariaatidega kohandamist. Analüüsides üksikuid bakteriliike tuvastati 133 statistiliselt olulist seost, millest ükski polnud statistiliselt oluline pärast mitmese testimise korrektsiooni. Kokkuvõttes võib öelda, et kohanemishäire puhul on muutusi soolestiku mikrobioomi koosluses.

Võtmesõnad:

Soolestiku mikrobioom, alfa-mimekesisus, beeta-mitmekesisus, psühhiaatrilised haigused

CERCS: Bioinformaatika, meditsiiniinformaatika, biomatemaatika, biomeetrika

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INTRODUCTION

Psychiatric disorder is a disease characterised by a pattern of behaviour causing distress or impairment of functioning. Globally, around 1 in 3 adults will experience a common psychiatric disorder within their lifetime (Steel et al., 2014). Psychiatric disorders can also lead to various comorbidities and are associated with deteriorating health.

Adjustment disorder (AD) is a mental disorder characterized by emotional or behavioural symptoms that develop within 1 month of a stressor. Stressors are usually a significant life change which may affect the individual's social network, financial supports or normal life (World Health Organization., 2004). AD is one of the most diagnosed mental disorders in psychiatric care and it is associated with high suicide rates. AD is a disorder with severe outcomes, high in prevalence and lack of research. Our study aimed to expand our understanding of AD by exploring the relationship between AD and the human gut microbiome.

In this study, the gut microbiome composition was analysed and compared between participants with AD and controls. As far as we know, there have not been any gut-brain-microbiome studies on AD.

1 LITERATURE REVIEW

1.2 Rise in prevalence of psychiatric disorders

Psychiatric disorder is a disease characterised by a pattern of behaviour causing distress or impairment of functioning. These behaviours can range from negative thoughts, lack of energy, difficulty concentrating, or emotional dysregulation. The most common psychiatric disorders include anxiety disorders, mood disorders and substance use disorder. Globally, around 1 in 3 adults will experience a common mental disorder within their lifetime and around 1 in 5 adults will meet criteria for a common mental disorder during 12 months preceding assessment (Steel et al., 2014). Thus, psychiatric disorders are highly prevalent and therefore have a major impact on the public health. Importantly, medical advancements are failing against psychiatric disorders and addictive disorders, which have increased by 4.3% and 16.3%, respectively between 1990 to 2016 in disability-adjusted life years (DALYs), which measures the number of years lived with disability and years of life lost from premature mortality (Rehm & Shield, 2019). Today, the most common disorders with high social impact, anxiety disorders and major depressive disorder (MDD) have global point prevalence around 7.3% and 4.7%, respectively (Baxter et al., 2013; Ferrari et al., 2013). Moreover, the prevalence of both anxiety disorder and MDD have risen drastically during the COVID-19 pandemic. During 2020, global prevalence of anxiety disorder and MDD increased by 25.6% and 27.6% respectively (Santomauro et al., 2021). This highlights the need for understanding the disease mechanisms and developing novel therapy options.

In addition to drastically affecting the quality of life of the individual, the psychiatric disorders can also lead to various comorbidities and are associated with deteriorating health. Mental disorders have been strongly linked with poor quality of life with frequent negative emotions, low energy and poor lifestyle (Connell et al., 2012). This leads to decreased life expect expectancy and increased risk of physical illnesses such as cancer, cardiovascular diseases and obesity due to making poor choices such as smoking and drinking, experiencing side effects from psychiatric medication and low energy to stay physically fit (Osborn, 2001). This furthermore highlight the need for early interventions that would also reduce the risk for comorbidities. However, only 0.8% to 15.3% of individuals with a psychiatric disorder or substance use disorder will seek treatment in a 12-month period, higher in more developed countries (Demyttenaere et al., 2004). Taken together, not only novel interventions, but effective prevention of the psychiatric diseases is desirable.

The Inequities of Mental Health Research Funding Report found global mental health research funding was around 3.6 billion US dollars per year but underfunded compared to other physical diseases. Since cancer and neoplasms research and infections research both received more than twice as much investment (Woelbert et al., 2020). Despite mental health having the highest years lived with disability (YLD) which may even be underestimated due to overlap with neurological disorders, conflation of all chronic pain syndromes with musculoskeletal disorders and exclusion of personality disorders. The true global burden of mental health could be 32.4% of total YLD (Vigo et al., 2016).

Funding disparity occurs within mental health funding. $1.66 billion USD per year goes into non-disease specific research, while substance use disorders and depression have the most funding over 1 billion USD per year, justified by having the highest YLD. However, this leaves other disorders underfunded such as suicide and self-harm ($60.7 million), Posttraumatic stress disorder ($57.2 million), eating disorder ($24.6 million) and personality disorder ($6.9 million) (Woelbert et al., 2020).

With the increase in prevalence and burden on both the individuals and society, there is a greater need for mental health research and support, especially underfunded mental disorders like adjustment disorder.

1.2 Adjustment disorder

Described in the International Classification of Diseases, 10th version (ICD-10) from the World Health Organization (WHO), adjustment disorder (AD) is a mental disorder characterized by emotional or behavioural symptoms that develop within 1 month of a stressor and do not persist for more than an additional 6 months after the stressor is no longer present (World Health Organization., 2004). Stressors are usually a significant life change which may affect the individual's social network such as (relationship break up, or death of a family member), financial supports such as (loss of job, or economic crisis) or normal life events such as (marriage, having children, or retirement). The symptoms must cause impairment in social or occupational functioning, must be excess of a normal and expected reaction to the stressors and would enter remission if the person adapts or the stressors are removed. The lack of clear symptoms means, individuals could experience depressive mood, anxiety, insomnia, hopelessness, lack of energy, suicidal thoughts, trouble concentrating. However, AD can only be diagnosed in the absence of another mental or behavioural disorder.

AD is considered a mild disorder/sub-syndromal condition. Nevertheless, the condition, similarly to depression and anxiety disorder, displays an increased risk for severe condition. In a longitudinal studies of trauma-exposed individuals, those with AD reported significantly worse outcomes relative to those with no psychiatric diagnosis but better outcomes compared with those diagnosed with other psychiatric disorders (O'Donnell et al., 2016). A study of AD in primary care found AD patients had higher mental quality-of-life scores than patients with MDD but lower than patients without mental disorder (Fernández et al., 2012). However, several studies found high suicide associations in AD (Casey & Bailey, 2011). In Denmark, (Gradus et al., 2010, 2015) two population-based studies with a total of 76,435 AD cases and 707,621 controls have been carried out. The 2010 study, each case was matched with 30 controls with the same gender and day of birth, also adjusted for depression diagnosis, marital status and income. While the 2015 study, adjusted for diagnosis of depression, anxiety and substance use disorder. Both studies found that AD had suicide rates 12 times higher than those without the disorder. This is strong evidence linking AD with suicidality. Worryingly, the interval between suicidal communication and act was found to be less than 1 month in AD, far less than MDD 3 months, bipolar disorder 10 months, and schizophrenia 47 months (Runeson et al., 1996). Similarly, a psychological autopsy of suicide study found that 15% had AD (Manoranjitham et al., 2010). Additionally, patients diagnosed with AD have an increased risk for other psychiatric disorders, such as MDD, substance abuse, schizophrenia, bipolar disorder, antisocial personality disorder and generalised anxiety disorder (Nancy Andreasen & Hoenk, 1982; O'Donnell et al., 2016). Thus, AD is still a serious disorder with potentially severe outcomes.

AD is one of the most diagnosed mental disorders in psychiatric care (Evans et al., 2013) and the prevalence in the general population is around <1% – 2% (Glaesmer et al., 2015; Gradus, 2017). In clinical settings or high risk groups, the prevalence rate is higher up to 35% (Okamura et al., 2000) and varies greatly between countries, populations and due to differences in diagnostic tools used. For example, a multisite study of consultation psychiatry services in the United States, Canada, and Australia found a 12% prevalence of AD, while a involuntarily unemployed sample had AD rates of 27% (Perkonigg et al., 2018; Strain et al., 1998) and bereaved sample had rates of 18% (Killikelly et al., 2019). An explanation for this high diagnostic rate, AD could be used as a waste basket diagnosis for patients to quickly get doctor's note for work, insurance or other non-medical reasons due to the lack of specific symptom profile (Patra & Sarkar, 2013). Nevertheless, it is clear that AD affects a large part of the population.

Despite high prevalence and increased risk for severe outcomes, AD has attracted few research studies and evidence based treatment. One reason is the lack of clarity in AD's criteria, making it difficult to distinguish between AD or other psychologic disorder such as depressive mood (Baumeister et al., 2009). Also, lack of guidelines to distinguish AD from normal adaptive reactions to stress (Casey & Bailey, 2011). A systematic review of psychological and pharmacological interventions for adults with AD. The psychological interventions included cognitive behavioural therapy, psychodynamic psychotherapy and relaxation-based therapy which are effective for treating general anxiety disorder and MDD (Leichsenring, 2001; Leichsenring et al., 2009). While the pharmacological interventions included selective serotonin reuptake inhibitor (SSRI) and benzodiazepine medication which are common and effective for anxiety disorders and MDD (Dunlop & Davis, 2008). However, relating to AD, the review had little confidence as to whether the observed effects are true effects of treatment due to inconsistent diagnosis with both The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) and ICD-10 diagnostic criteria, lack of baseline clinician-administered assessment, lack of follow-up assessment, small sample sizes, lack of controlling for antidepressants or other medications (O'Donnell et al., 2018). Another review also concluded a lack of empirical evidence for psychological treatments of AD and only finding one intervention for adolescents(Jojic & Leposavic, 2005). Review also raise the concern of trials without a no-treatment control group, meaning observed benefits could be due to the removal of the stressors (Domhardt & Baumeister, 2018).

Due to the high prevalence, high suicide association and the lack of research, our study aimed to expand our understanding of AD by exploring the relationship between AD and the human gut microbiome.

1.3 Microbiome, it's role in human health

The human gut microbiome (GM) is composed of all the microorganisms (archaea, bacteria, eukaryotes, fungi, and viruses) in the human gastrointestinal tract (Dave et al., 2012). There is an rough estimate of 1000 bacterial species in the gut, which consist of 100 times more genes than in the human genome (Turnbaugh et al., 2007). Unlike the human genome which is >99% identical across all humans, the microbiome is vary widely between healthy people (Huttenhower et al., 2012). The GM composition and diversity is heavily influenced by the person's environment from the moment of birth. For example, natural and caesarean-section infants have different GM profiles (Shao et al., 2019). Throughout the lifespan, the GM

grows in diversity and has taxonomic differences between life stages (Badal et al., 2020). Major factors determining the GM composition are gastrointestinal (GI) tract physiology, diet, environment and geography, while factors with most profound effects are antibiotic usage and extreme diet changes (Dave et al., 2012). A healthy and diverse GM is important for digestion by providing nutrition from indigestible substrates to the host and synthesize vitamins. Also, helps immunity by protecting against pathogens, and support proper immune response (Thursby & Juge, 2017).

Due to the already identified biological mechanisms and functional potential of the microbiome, the microbiome research has focused on studying the role of gut microbiome in complex diseases. Already, the disruption of GM has been associated with numerous diseases such as irritable bowel disease, bacterial infections and inflammatory bowel disease (IBD). More research are linking the influence of gut microbiome on common diseases such as obesity, rheumatoid arthritis, colorectal cancer, diabetes, cardiovascular disease, and even nervous system disorders (Shreiner et al., 2015). The identified associations show that the microbiome can have a use in clinical applications. For example, the microbiome is being used for improving the detection of colorectal cancer (Wirbel et al., 2019).

The gut microbiome is distinct from the human genome primarily because of the possibility to rather easily modify the microbial composition. Thus, treatment and prevention interventions which alter the GM can be also used to ameliorate disease symptoms. Non-medical interventions such as diet and exercise can help maintain homeostasis or increase the diversity of the GM. Faecal microbiota transplantation (FMT) involving transplantation stool from a healthy donors to patients can rapidly alter the GM. FMT has a clinical resolution rates of up to 93% for relapsing *Clostridium difficile* infection making it by far the best treatment for the condition (Nie et al., 2019). Furthermore, GM can potentially be used a disease marker for prediction or classification with enough longitudinal studies monitoring the GM before and after disease onset. For example, Kostic et al. monitored the relationship between GM and type 1 diabetes (T1D), founding T1D progression was aligned with decrease in alpha-diversity and increase in inflammation-favouring organisms, gene functions, and serum and stool metabolites (Kostic et al., 2015).

Although we are still only beginning to understand the relationship between GM and disease, it is clear that gut microbiome harbours great potential and properties for it to be used in clinical applications. However, the focus of microbiome studies is heavily shifted towards gastrointestinal disorders and metabolic disorders. The work on psychiatric disorders and

adjustment disorder is scarce. Thus this thesis focuses on understanding the GM composition of individuals with AD.

1.4 Microbiome in psychiatric disorders and adjustment disorder. Gut-brain-microbiome axis.

The main biological mechanism that assumes an association between microbiome and psychiatric disorders and motivates the ongoing research is referred to as the gut-brain-microbiome (BGM) axis. The BGM axis is a neural network which allows a bilateral interaction between the gut microbiome and the central nervous system (CNS) with neuroimmune and neuroendocrine mechanisms, and via the vagus nerve. Shown on figure 1, The vagus nerve starts from medulla oblongata connects to the stomach, small and large intestines where the bidirectional BGM communication occurs (Breit et al., 2018).

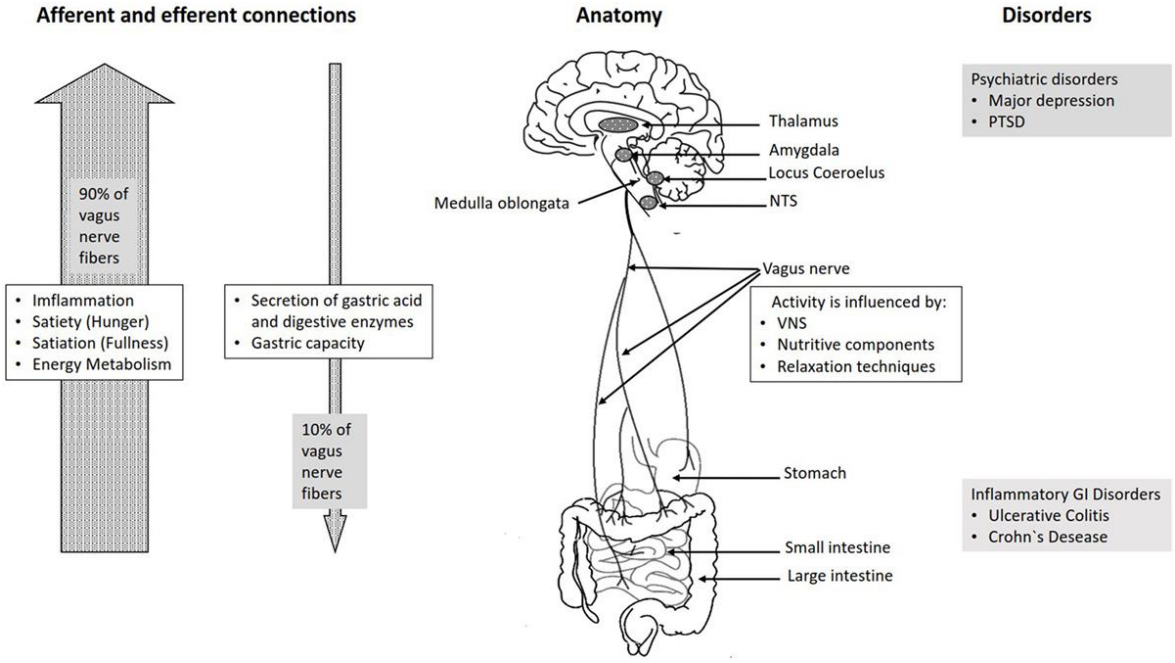

Figure 1. Overview over the basic anatomy and functions of the vagus nerve (Breit et al., 2018).

Different types of cells making up the endocrine system of the gut can release signalling molecules in response to chemical and or mechanical stimuli. These molecules can enter the systemic circulation and reach the CNS or act locally on the vagus nerve regions in the gut or liver (Martin et al., 2018).

Animal models have shown the association between GM and psychiatric disorders. For example, germ-free mice have higher exaggerated stress-induce hypothalamic-pituitary-adrenal axis response compared to control mice, which was reversable by providing

Bifidobacterium infantis (Sudo et al., 2004). This means that a healthy GM is needed for normal brain function and specific bacteria strain could be used as medication for psychiatric symptom. A study transferred human faecal microbia from depressed individuals into microbiota-deficient rat model. The rats developed characteristic of depression including anhedonia, anxiety-like behaviours and alterations in tryptophan metabolism (J. R. Kelly et al., 2016). Thus, showing certain bacteria could be causing symptoms of depression and anxiety. Also, early life stress in rats showed association with increased intestinal permeability and bacterial translocation to the liver and spleen (Moussaoui et al., 2014). Increase in intestinal permeability means that bacteria and compound in the gut can pass through the gut barrier and into the blood stream. Allowing gut bacteria can move to different parts of the body. This can cause inflammation when the immune system is activated to fight the foreign bacteria. Interestingly, systemic inflammation has been linked to depression (Obrenovich, 2018). This shows a potential mechanism how even past stressful events in adjustment disorder could disrupt the GM.

Human models have also been used to support the BGM axis interaction. For example, Autism spectrum disorder (ASD) has been linked to the gut microbiome (Vuong & Hsiao, 2017). Faecal transplant from healthy children to children with ASD resulted in significant improvement in both gastrointestinal (GI) Symptoms and ASD Symptoms (Li et al., 2021). One study showed that acute-stress significantly increased intestinal permeability in students with elevated cortisol (Vanuytsel et al., 2014). Soldiers under prolonged physical stress also showed increased intestinal permeability and correlation with changes in *Bacteroidetes* and *Actinobacteria* (Philip Karl et al., 2017). These studies again shows the potential mechanism how stress can alter the GM by causing increased intestinal permeability. Many common psychiatric disorders and symptoms have been linked with the gut microbiome or GI problems. Thus, the research suggest treatment through the BGM axis interaction is possible.

Gut interventions to ameliorate psychiatric have been studied in rodents. Studies have found that probiotics could reverse intestinal permeability changes from stress and decrease anxiety and depression symptoms (Ait-Belgnaoui et al., 2012). *Lactobacillus reuteri* treatment reversed the social deficits in ASD model mice and germ-free mice via the vagus nerve (Sgritta et al., 2019).

In humans, there are mixed results. A schizophrenia study found that probiotic intervention was no better than a placebo (Dickerson et al., 2014). A meta-analysis of anxiety and depression clinical-trails found that prebiotics did not different from placebo but probiotics

had small but significant benefits in both disorders. Two successful prebiotic strains from mouse models were studied in humans. *Bifidobacterium longum* 1714 prebiotics reduced stress and improved memory in healthy humans while *Lactobacillus rhamnosus* failed (Allen et al., 2016; J. R. Kelly et al., 2017). Nevertheless, there are relatively few studies that lack after-treatment follow-up evaluation and include only adult participants. Hence more clinical-trials are needed to fully evaluate the efficacy of microbiome-based interventions (Liu et al., 2019). This shows the need for human trials because not all animal model results translate to humans.

Many other concerns are present in BGM-related research. First, meta-analysis showed that large proportion of BGM-related studies are focused on psychosis and schizophrenia disorders, mood disorders, anxiety disorders and eating disorders. Meanwhile, AD in the stress-related disorder category only has one study on PTSD. The mean sample size in the meta-analysis was only 45 patients per study, which is considerably small (Nikolova et al., 2021). Another issue is that most studies are cross-sectional. Healthy humans have daily changes to their microbiome composition, that can lead to false discoveries (Koren et al., 2013). Longitudinal studies or at least follow-ups should be used to find interesting microbiome changes. For example, (Bjørkhaug et al., 2019) found the genus *Holdemania* to be elevated in individuals with chronic alcohol overconsumption in their cross-sectional study, while (Leclercq et al., 2014) found decreasing abundance of *Holdemania* from 3 weeks of alcohol withdrawal. Longitudinal studies allow us to observe the BGM interaction overtime. Furthermore, studies have analysed differences in

taxonomic composition at different taxonomic ranks. The best option is to analysis at the most lowest taxonomic level because for example, species of the same genus can be negatively correlated and carry different functional potential. For example, depression symptoms in females was found to be positively associated with C. *citroniae* but negatively associated with C. *innocuum* and C. *hathewayi* (Ganci et al., 2022). There is also a lack of consensus on which type of analysis method to use in microbiome analysis. For example, Nearing et al used 14 different differential abundance methods and produced different results on the same dataset. They concluded that ALDEx2 and ANCOM-II produce the most consistent results across studies (Nearing et al., 2022).

Our study is to explore the gut microbiome composition of participants with AD. As far as we know, there have not been any BGM studies on adjustment disorder.

2 THE AIMS OF THE THESIS

The aim of the thesis was to analyse, whether the gut microbiome composition is associated with adjustment disorder. The specific aims were the following:

- Analyse the gut microbiome composition between participants with adjustment disorder and controls.
- Find bacterial taxa association with adjustment disorder

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Estonian biobank

This thesis uses the gut microbiome data, which is part of the Estonian biobank data collection. The Estonian biobank (EstBB) is a volunteer-based cohort of the adult population started in 1999. The objective was to investigate the genetic, environmental and behavioural background of common diseases and traits by creating a biobank with biological samples and health records from a large proportion of the population (Leitsalu et al., 2015). The biobank is supported by the Estonian Human Genes Research Act (HGRA), which allows the collection of genetic information to be used in genetic research to improve public health. Participants are recruited by general practitioners (GPs) and medical personnel in the special recruitment offices. Over 200,000 participants have been so far recruited to EstBB. As part of the EstBB, the Estonian Microbiome cohort (EstMB) was established and

2,509 EstBB participants were recruited for a follow-up data collection. The EstMB participants provided stool, oral, and blood samples and filled in self-reported questionnaires about lifestyle.

3.1.2 Microbiome sample collection and sequencing

The participants collected a fresh stool sample immediately after defecation into a polypropylene conical 15-mL tube and delivered it to the study centre, where it was stored at -80 °C. Microbial DNA extraction was performed using QIAamp DNA Stool Mini Kit. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA The DNA sample was fragmented by sonication to an average size of 350bp, amplified using polymerase chain reaction and Shotgun metagenomic paired-end sequencing was performed by Novogene Bioinformatics Technology Co., Ltd. Using the Illumina NovaSeq6000 platform. Reads were trimmed for quality and adapter sequences. The host reads that aligned to the human genome were removed using SOAP2.21. The taxonomic composition of the metagenomes was identified by comparing the marker gene homologs to a NCBI nonredundant NCBI-nr (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) database (201810) of taxonomically informative gene families using the DIAMOND software (v0.9.9.110).

3.1.3 Defining cases and controls

The AD cases consisted of 236 participants with at least one adjustment disorder diagnosis labelled as F43.2 based on the ICD-10 medical classification in the national electronic health records (EHRs). Only diagnosis by medical professionals was used. All subtypes of adjustment disorder labelled with 4 character codes, for example F43.22 are considered as a F43.2 diagnosis. The AD diagnosis ranged from 1998 to 2019 (Supplementary figure 2). The other 2270 participants were included in the control group.

3.1.4 Statistical analysis of microbiome data

All statistical analyses were carried out using R (v.4.1.3) software and Rstudio (2022.02.1 Build 461)

We analysed the differences in microbiome composition between the cases and controls using two strategies. Initially, we looked for differences on the community-scale using diversity analysis after which we run univariate analysis for each species separately. All of the analysis were adjusted for the age, body mass index (BMI) at the time of stool sampling, antibiotic usage within 6 months before stool sampling, the gender of the participant and usual stool type that was simplified 3 groups into constipation, normal or diarrhea (Supplementary Figure 1).

These covariates are significantly associated with differences in the gut microbiome composition. The difference of means between the two groups were analysed using Welch Two Sample t-test and differences in the distributions of discrete variables were analysed using Pearson's Chi-squared test with Yates' continuity correction from base R.

3.1.4.1 Alpha diversity

Alpha diversity is an ecological concept that aims to characterize the within-sample diversity of an ecosystem. The two metrics of alpha-diversity can be broadly categorised into richness and evenness. Richness of the ecosystem measures the total number of unique species. On figure 2, sample A has lower richness compared to sample B because it only has 2 unique bacteria while sample B has 4 unique bacteria. Evenness measures how evenly are the abundances of the bacteria distributed within the ecosystem. Again on figure 2, sample B has higher evenness compared to sample C because the bacteria are even in abundance while sample C is predominantly a single species.

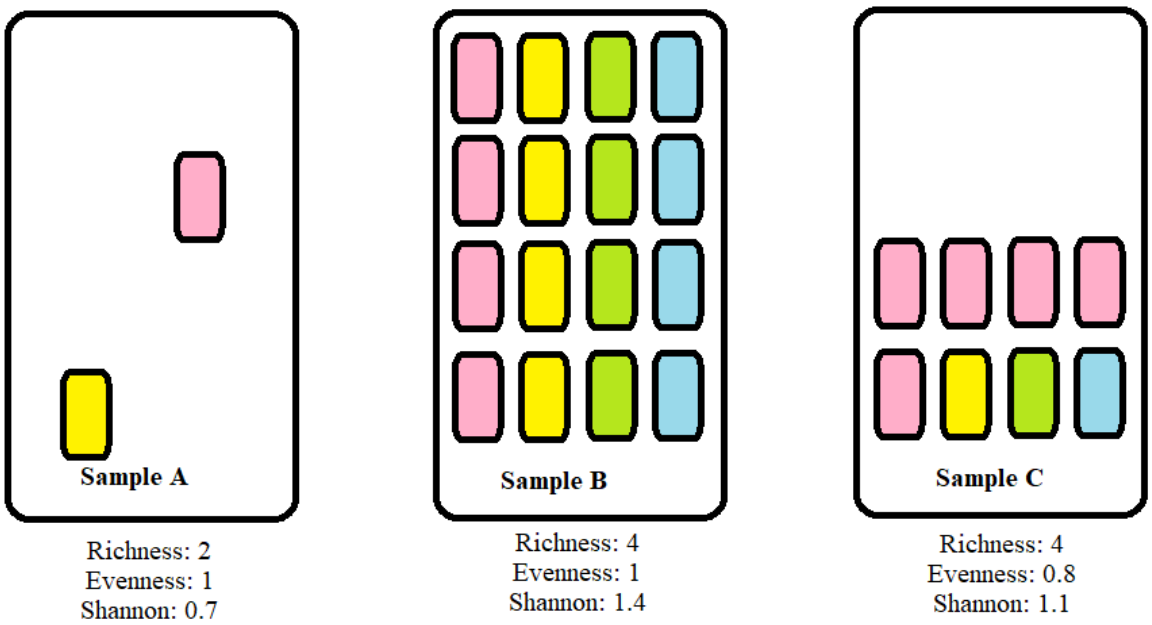

Figure 2. Illustration of the richness and evenness of each community and shannon diversity index score of each.

Shannon's index diversity was used to calculate alpha diversity from the vegan package (2.6-2). Shannon index takes into account both richness and evenness.

$$H = -\sum[(p_i) \times \ln(p_i)]$$

H – Shannon diversity index

p_i – relative abundance of the i-th species in the whole community

Simple linear regression model was performed to analyse the alpha diversity between case/control groups, while multiple linear regression model was used when adjusting for covariates. This provides understanding if a disease or other factor is associated with differences in alpha diversity.

3.1.4.2 Beta diversity

Beta diversity quantifies the differences in the species composition between two samples. On figure 3, sample A and sample B are similar in bacteria species hence it's given a low score of 3. While sample A and sample C are highly dissimilar, they do not have the same bacteria species hence it's given a high score of 5. All samples are compared and score between each other. To visually compare the differences between the microbiome composition of cases and controls, principal coordinates analysis (PCoA) was made. The idea of PCoA analysis is to reduce the dimensionality of the dataset and to represents the samples on a 2 dimensional plot. Each point is a single sample, and closer the points are the more similar

their microbiome profiles are. This allows beta diversity of all the samples to visualised and compared on a single plot. However, the process of reducing the dimensionality only takes in the most important variation to generate the plot, which means not all the variation between samples are used hence statistical testing methods are used to fully test beta diversity differences.

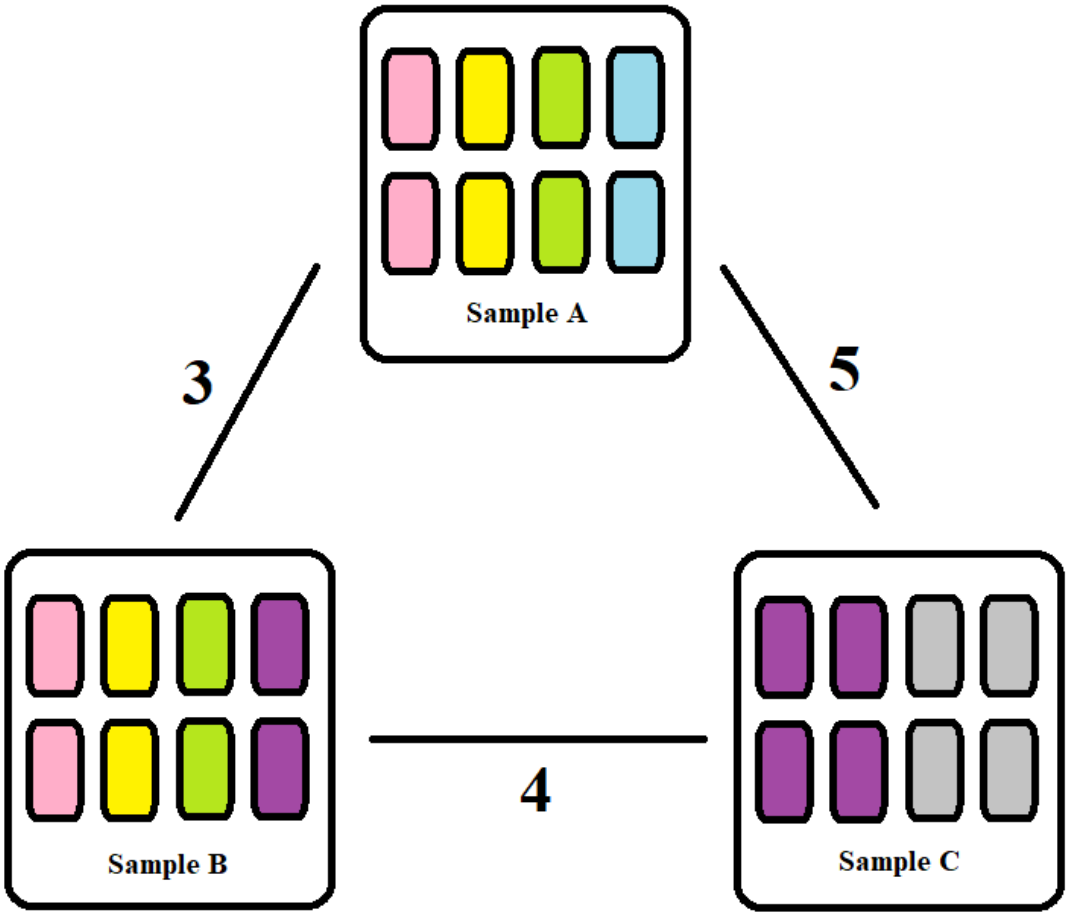

Figure 3. Illustration of beta diversity comparison. Higher score shows dissimilarity of the microbiome composition between two samples.

A Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) was carried out to test the differences in the microbiome composition between AD group and controls. Bray-Curtis index was used as a distance measure, which takes in account the number of species and their relative abundance differences of the samples. To calculate the p-value, 5000 permutations were used.

3.1.4.3 Differential abundance analysis

Differential abundance analysis aims to identify if there is a difference in the abundance of individual taxonomic group between two groups or more. It can provide information about if the bacteria abundance is higher or lower between the groups and can find correlation between a bacterial taxa and a disease. However, causation cannot be confirmed without longitudinal studies and controlling for covariates. In this study, ALDEx2 package was used which tests individual taxonomic group abundance differences using Wilcoxon Rank Sum test and Welch's t-test. It reports both p-value and Benjamini-Hochberg corrected p-values to reduce false discovery rate.

3.2 **RESULTS**

3.2.1 Descriptive analysis of the study cohort

A total of 2509 participants were part of the study but 3 participant were removed due to poor stool quality results. Plus, there were missing data, 18 without age, 3 without BMI and 71 without stool type. There were 236 participants (10.4%) who have been diagnosed with AD. The mean age of AD participants (47.5 years) were significantly lower than control (50.3 years). The percentage of women were significantly higher in AD (79.7%) than control (69.3%). While, differences in BMI, antibiotics use and stool type were non-significant, despite antibiotic use being 6.5% higher in AD participants (Table 1).

Table 1

Characteristics of the Study Cohort

	Adjustment disorder (n = 236)	Control (n = 2270)	P-value
Age*	47.5 ± 13.5	50.3 ± 15.1	0.003
BMI**	25.9 ± 5	26.5 ± 5.4	0.0503
Sex (Female)	188 (79.7%)	1573 (69.3%)	<0.001
Antibiotic use	57 (24.2%)	425 (18.7%)	0.054
Stool type***			0.619
Normal	137 (58.1%)	1268 (55.9%)	
Diarrhea	39 (16.5%)	431 (19%)	
Constipation	55 (23.3%)	505 (22.2%)	

Age in years when the stool sample was collected. BMI: Body Mass Index. Antibiotic usage within 6 month before stool sampling. Stool type is the most frequent type for the individual. Data is represented as the mean ± standard deviation or as a number (percentage) Difference of mean calculated using Welch Two Sample t-test and for discrete variable calculated using Pearson's Chi-squared test with Yates' continuity correction
*4 AD and 14 control without age data
**3 control without BMI data
***5 AD and 66 control without stool type

3.2.2 Alpha diversity

Alpha diversity was calculated using Shannon index and the distribution for AD and control are represented on figure 4. The alpha diversity seen to be significantly higher.

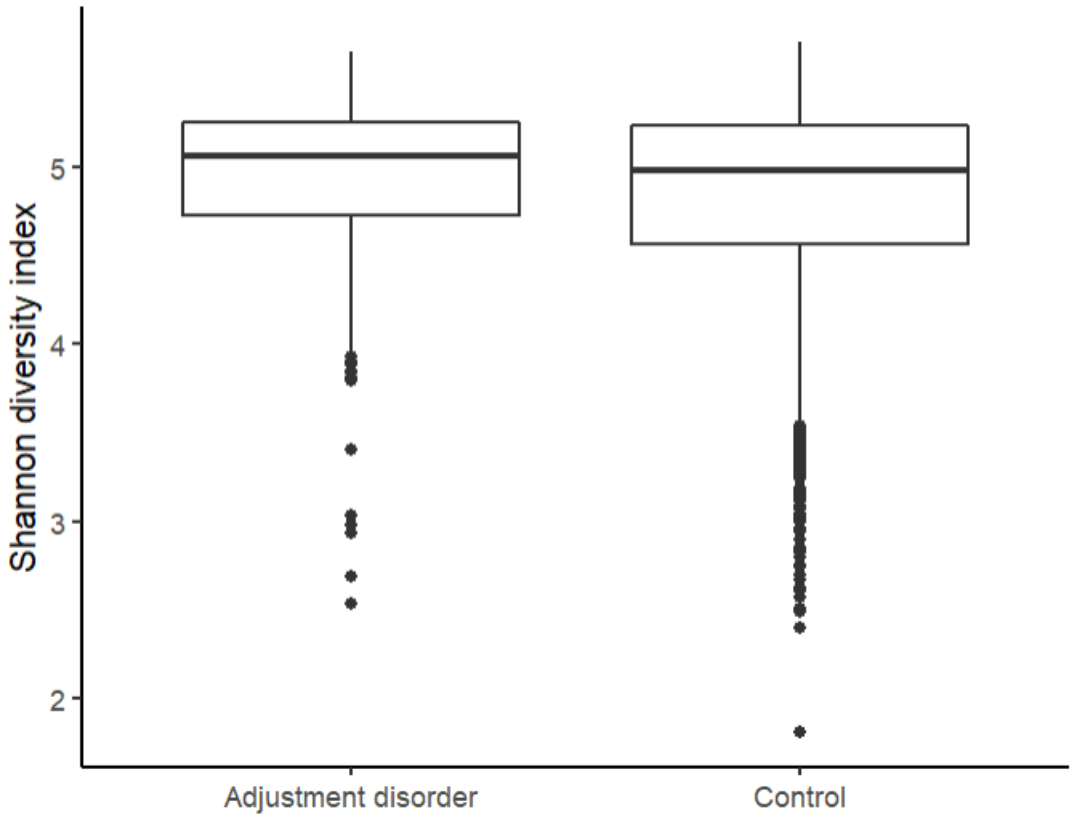

Figure 4. Difference between adjustment disorder and control in alpha diversity measured by Shannon diversity index. The medium is represented by the line inside the box, the straight line represent the lowest and highest values within the 1.5 interquartile range and the dots represent outliers.

In order to confirm if the alpha diversity is significantly higher in AD, a linear model was used. The results are shown in table 2, The Shannon index was significantly (p = 0.0213) higher by 0.0908 in AD group compared to controls without covariate adjustment.

Table 2

Alpha Diversity comparison Adjustment Disorder (AD) and Control

	β-coefficient	Standard error	P-value
Intercept	4.8213	0.0121	<0.0001
AD	0.0908	0.0394	0.0213

Linear regression model used to calculate the adjustment disorder coefficient to Shannon index.

In order to adjust for covariates and reducing confounding, multivariate linear regression model was used and the results are shown on table 3. However, the significant difference was lost (p = 0. 09607) and 81 participants were removed due to missing data. The covariates

adjusted were age when the stool sample was collected, antibiotic usage within 6 month before stool sampling, BMI, gender and the most frequent stool type for the individual. The covariates were all significantly correlated with alpha diversity differences, with BMI, gender and stool type being highly statistically significant in the model. Being older, being male, frequent constipation were associated with higher alpha diversity, while antibiotic usage, higher BMI and frequent diarrhea were associated with lower alpha diversity. In the model only AD has an adjusted R^2 of 0.0017 while with covariates it was 0.1154.

Table 3

Alpha Diversity comparison Adjustment Disorder (AD) and Control with covariate adjustments

	β-coefficient	Standard error	P-value
Intercept	5.1859	0.0664	<0.0001
AD	0.0622	0.0376	0.0983
Age*	0.0018	0.0008	0.0190
Antibiotic	-0.0898	0.0278	0.0013
BMI**	-0.0137	0.0022	<0.0001
Gender (Male)	0.1756	0.0244	<0.0001
Stool, Diarrhea	-0.4467	0.0342	<0.0001
Stool, normal	-0.1875	0.0272	<0.0001

Age in years when the stool sample was collected. BMI: Body Mass Index. Antibiotic usage within 6 month before stool sampling. Stool type is the most frequent type for the individual (Constipation, Normal or diarrhea). Linear regression model used to calculate each variable coefficient to Shannon index.
*4 AD and 14 control without age data
**3 control without BMI data
***5 AD and 66 control without stool type

3.2.3 Beta diversity

To visually compared the microbiome composition between AD and control, we produced a PCoA plot as seen on figure 4. The first principle component explains 19% of the variation while the second explains 13% meaning only 32% of the variation in beta diversity is covered by the plot. The AD participant samples are shown in red and control in green. The AD participants seems evenly spread out meaning we are unable to observe any patterns which show a differences between AD and control.

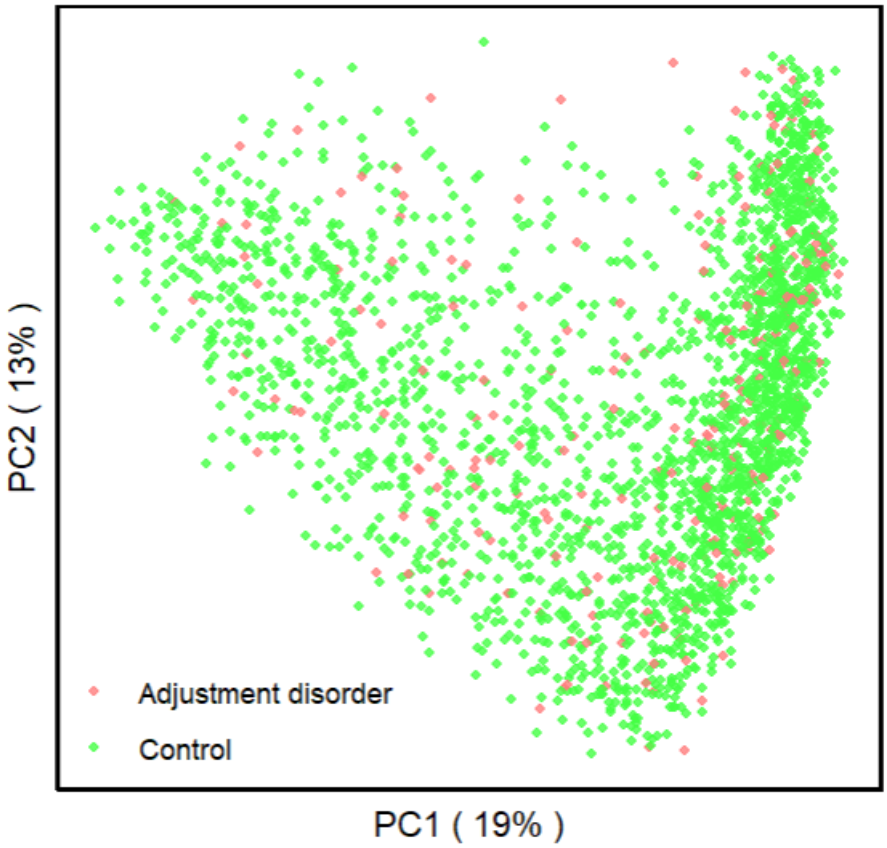

Figure 5. Characterisation of beta diversity between Adjustment disorder (AD) and control. Bray-Curtis index calculates the dissimilarity of the microbial community between every sample then Principal coordinates analysis (PCoA) reduces the dimensionality of the data, and presents each sample with two principle component. First principle component (PC1) explains 19% of the variation while the second (PC2) explains 13% AD group is red and control group is green.

In order to test the differences in beta diversity between AD and control, we ran PER-MANOVA using Bray-Curtis index. The results of the first beta diversity analysis without covariates are shown on table 4. The analysis showed that the microbiome composition was significantly different in AD group compared to controls (P = 0.0096, R2 = 0.0009).

Table 4

Beta Diversity Between Adjustment Disorder and Control

	Residual sum of squares	R^2	F-value	P-value
AD	0.44	0.0009	2.226	0.0096
Residual	499.94	0.9991		

Beta diversity calculated with Permutational Multivariate Analysis of Variance Using Distance Matrices

The second analysis adjusted for covariates and the results are shown on table 5. The beta diversity was still significantly different with (P = 0.017, R2 = 0.0008). The R^2 dropped from 0.0009 to 0.0008. A small part of the beta diversity difference in AD was due to confounding. All covariates were highly significant and have larger R^2 compared to AD. Stool type had the highest R2. The residual had a large R^2 of 0.9647.

Table 5

Beta Diversity Between Adjustment Disorder and Control

	Residual sum of squares	R^2	F-value	P-value
AD	0.4	0.0008	2.073	0.017
Age*	1	0.0021	5.182	0.0002
Antibiotic	1.53	0.0032	7.901	0.0002
BMI**	3.64	0.0075	18.840	0.0002
Gender (Male)	2.65	0.0055	13.720	0.0002
Stool type***	7.87	0.0162	20.347	0.0002
Residual	467.18	0.9647		

Age in years when the stool sample was collected. BMI: Body Mass Index. Antibiotic usage within 6 month before stool sampling. Stool type is the most frequent type for the individual (Constipation, Normal or diarrhea). Beta diversity calculated with Permutational Multivariate Analysis of Variance Using Distance Matrices
*4 AD and 14 control without age data
**3 control without BMI data
***5 AD and 66 control without stool type

3.2.4 Differential abundance analysis

In order to find the specific differences found in beta diversity analysis. We ran differential species analysis using Welch's T-test and Wilcoxon-test with Benjamini-Hochberg (BH) procedure to detect the bacterial taxa, which have abundance difference between AD and control. In the entire cohort, 1231 different bacteria were detected up to subspecies taxonomic level but some sequence reads could only classify to phylum level. We used the standard ($p < 0.05$) and detected 133 significant differences in abundance between AD and controls with the same covariate adjustments. Table 6 shows the top ten most significant bacterial taxa.

Table 6

Differential Species Analysis between Adjustment Disorder and Control

Bacteria	Taxonomic level	Estimate	P-value	BH P-value
Clostridium sp. CAG:7	Genus	0.412	<0.0002	0.190
Oscillospiraceae VE202-24	Family	0.298	<0.0002	0.228
Bacteroides sp. OM08-17BH	Genus	0.484	<0.0002	0.233
Firmicutes CAG:466	Phylum	0.224	0.0004	0.503
Anaerotignum lactatifermentans	Species	0.291	0.0006	0.727
Oscillibacter sp. KLE 1728	Genus	0.283	0.0006	0.75
Oscillibacter sp. KLE 1745	Genus	0.379	0.0007	0.779
Alistipes onderdonkii	Species	0.435	0.0013	1
Flavonifractor sp. An10	Genus	0.205	0.0015	0.998
Bacillus cereus	Species	0.219	0.0019	0.999

The top ten most significantly different bacteria taxa abundance between AD and control. Taxonomic level is the lowest classified. Difference of mean calculated with a two sample Welch's T-test and Wilcoxon-test. Calculated both P-value ($p < 0.05$) and P-value with Benjamini-Hochberg procedure.

The top ten bacteria all had positive estimate values, meaning there were found to have higher abundance is patients with AD compared to controls. At the phylum level, eight out of ten were from *firmicutes*, while *bacteroides* sp. OM08-17BH and *alistipes onderdonkii* were from *bacteroidetes*. At family level, the associated species showed a diverse range including *clostridiaceae, oscillospiraceae, bacteroidaceae, lachnospiraceae, rikenellaceae,* and *ruminococcaceae*.

However, no significant differences were detected after applying Benjamini-Hochberg (BH) procedure as shown on table 6. The BH procedure is applied to correct for multiple testing and decrease the rate of false discovery.

3.3 DISCUSSION

This thesis aimed to compare the subjects with AD and control to find differences in their microbiome composition. The percentage of AD in the Estonian microbiome was 10.6%, which is higher than the general population of 1-2% (Glaesmer et al., 2015; Gradus, 2017). The reason for this is unknown and should be explored in another study. The higher percentage of women in AD group was also expected because women are more likely to be diagnosed with AD compared to men (Gradus et al., 2015). Overall, the characteristics of the study cohort was in line with expectations.

For alpha diversity, we compared the difference using Shannon index between AD and control to see if there was a difference. We observed significantly higher alpha diversity in AD group. This is surprising because (Nikolova et al., 2021) found lower alpha diversity in patients with mental disorder diagnosis when compared to healthy controls, suggesting alpha diversity is negatively associated with mental disorders. Although, it aligns with findings from (L. S. Kelly et al., 2021), where mice who have experienced trauma and experience ongoing stress had increased alpha diversity overtime. An explanation could be the increased intestinal permeability due to acute-stress, which has shown to alter the microbiome composition in humans (Philip Karl et al., 2017). Although, confounding factors could contribute to this difference because AD diagnosis was no longer significant after adjusting covariates such as BMI, gender, antibiotic usage and stool type. These covariates were all significantly associated with alpha diversity as BMI, gender and usual stool type were highly significant in the model. The covariate results align with current knowledge (Dominianni et al., 2015). As expected, the p-value was much lower compared to the covariates because we are including all participants who had a diagnosis of AD, not participants currently experiencing AD. It could also be that the correlation between alpha diversity and AD is low and unable to be detected with the current sample size. We couldn't confirm if the correlation detected would be directly related to AD because AD diagnosis are higher in clinical settings and high risk groups hence it could be an unknown confounding factor. Also, this study didn't include diet and lifestyle information, other medication besides antibiotics nor comorbidity due to the lack of power or lack of information. Medication is especially important for AD, which are usually medication usually for mood and anxiety disorders (Stein, 2018). Further research should include lifestyle and medical information such as diet, living situation, exercise level, hospital stays, medical history and medication to avoid confounding with AD.

For the beta diversity analysis, we are unable to observe any obvious clustering in the PCoA plot. The difference could be too small to detect and PCoA ignores information in the dimension reduction process hence we ran PERMANOVA to fully use all the beta diversity variation. The beta diversity was significantly different, even with covariate adjustments meaning the gut microbiome composition is significantly different between AD and control. Beta diversity differences have been observed in other psychiatric disorders such as major depressive disorder, psychosis and schizophrenia, however not in anxiety disorder which is similar to AD (Nikolova et al., 2021). The R^2 was relatively smaller than all other covariates at 0.0008 while age the second smallest had 0.0021 and stool type was the highest at 0.0162. This shows that AD diagnosis accounted for a relatively small difference in gut microbiome

compared to the covariates. Also, the residual R^2 was 0.9647, meaning the majority of the variation in the microbiome composition is unexplained by these covariates. Future research should aim to have gut microbiome data from individuals suffering from adjustment disorder. Although, we are more confident that the differences are not due to age, antibiotic use, BMI, gender and stool type since they were adjusted for. However, same issue with alpha diversity, we still lack other important factors diet and lifestyle, medication nor comorbidity. The hospital stays records are important for beta diversity because AD prevalent is higher in clinical settings and even short-term stay in hospitals can alter the bacteria taxa abundance (Zheng et al., 2021). These are possible confounding factors which future research should include.

For the differential abundance analysis, there were 133 bacterial taxa which were significantly different in their abundance. Interestingly, the top ten most significantly associated were all higher in AD. These could potential be used as diagnostic or risk evaluation markers for AD. They may even have potential physiological mechanism which attribute to AD symptoms. We found interesting associations from other research papers.

Clostridium sp. CAG:7 was positively associated and had the highest significance. This finding aligns with two animal models studies in gorilla and mice exposed to stress that also showed positive association in genus *Clostridium* (Bailey et al., 2011; Vlčková et al., 2018).

Two *Osillibacter* subspecies (KLE 1728, KLE 1745) were positively associated with AD. An possible explanation, *Osillibacter*'s main metabolic end is valeric acid, which can bind to gamma-aminobutyric acid (GABA) receptors due to similar structure. The GABA has a major role in regulating stress response. Other BGM studies, (Jiang et al., 2015; Naseribafrouei et al., 2014) both found positive association of *Osillibacte* in humans with MDD. GABA has a role in the pathology of depression (Kalueff & Nutt, 2007). In terms of treatment, a systematic review of oral GABA supplements in human trials have shown very little evidence for stress reduction benefits (Hepsomali et al., 2020). This supports the possibility of future research, which aims at reducing valeric acid producing microbes such as *Osillibacter* thought diet or medication to reduce symptoms of stress and depression.

Alistipes Onderdonkii was positively associated with AD. One autism BGM study found lower relative abundance of *Alistipes Onderdonkii* compared to controls in Autism but higher relative abundance in a milder form of Autism (de Angelis et al., 2013). Studies at genus level, the same MDD studies by (Jiang et al., 2015; Naseribafrouei et al., 2014) found positive association of *Alistipes* at genus level in humans with MDD.

Although there are 133 bacterial taxa which are significantly in abundance. This study cannot confirm the association because all the significant were eliminated after correcting for multiple testing meaning there is a high potential for false positives findings. The association could have been weak due to the low statistical power or covariates which can't be accounted for. Another explanation is that the gut microbiome differences do not persist after adjustment disorder remission.

In conclusion, there is suggestive evidence that the gut microbiome composition is associated with AD based on the significant differences in the GM diversity. In addition, there were bacterial taxa found which are associated with similar psychiatric disorders or AD symptoms. However, we couldn't confirm if the association detected would be directly related to AD until future research utilising medication history, diet and lifestyle information, comorbidity and hospital stay will be carried out to rule out possible confounding factors. Also, future longitudinal studies which analyse the GM composition of individuals experiencing adjustment disorder after when the stressors are removed are needed to pinpoint the possible causal microbial signals.

SUMMARY

Gut-brain-microbiome axis has been recognized as the main biological mechanism that supports the research linking microbiome and psychiatric disorders. Psychiatric disorders are a major issue in public health and highly prevalent globally.

Adjustment disorder (AD) despite high prevalence and increased risk for severe outcomes has attracted few research studies, which leads to the lack of understanding about the disorder. As far as we know, there have not been any gut-brain-microbiome studies on AD.

This research investigated potential gut microbiome composition associated with AD. The alpha diversity of AD was found to be significantly higher compared to controls and beta diversity showed that the gut microbiome composition between AD and controls is significantly different. To reduce confounding, the analysis was adjusted for covariates including age, BMI, antibiotic usage, gender, usual stool type. Differential abundance analysis found 133 bacterial taxa abundance significantly different in AD. The top ten had higher abundance in AD compared to controls. An observable difference in diversity and bacterial taxa abundances suggest that the gut microbiome is associated with AD, but further research is needed to understand the potential mechanisms and causality.

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Supplementary

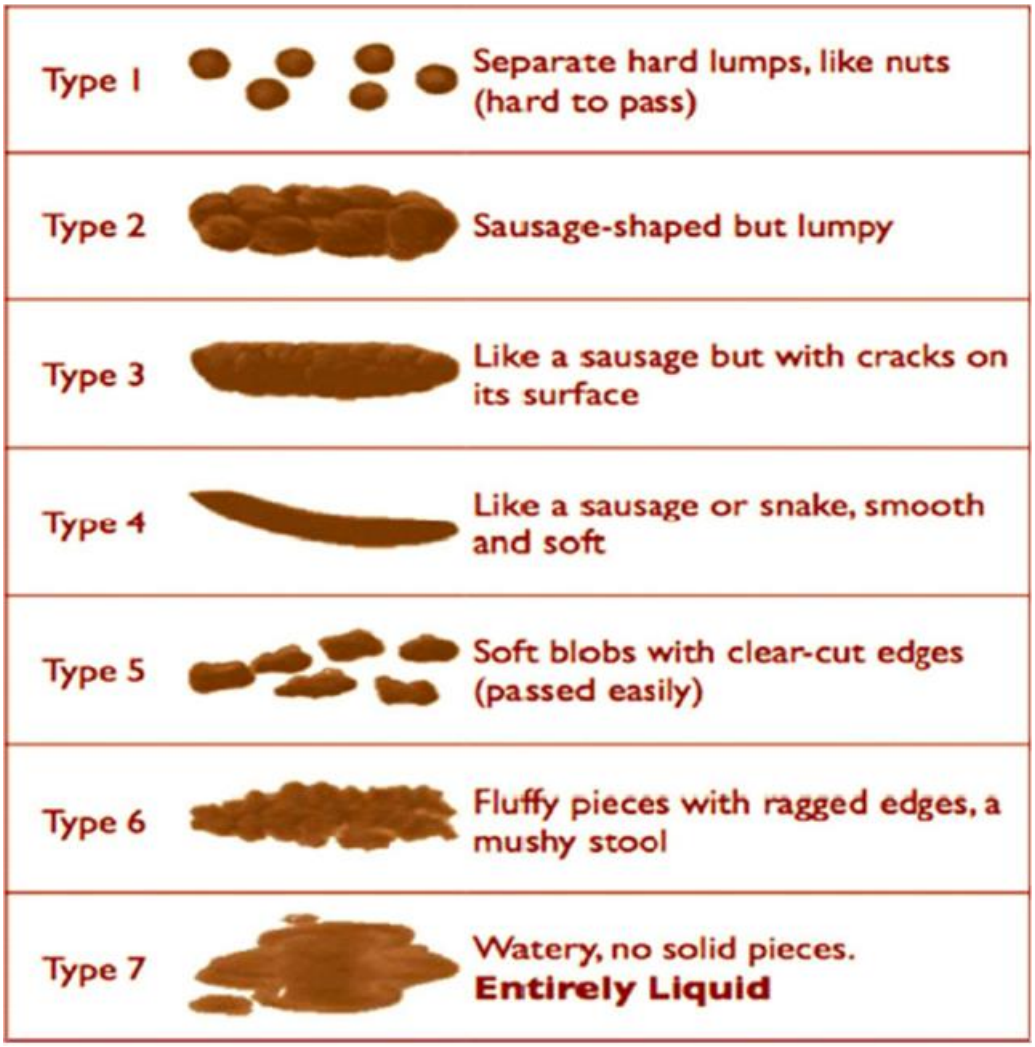

Figure 1. The usual stool type of the cohort from the Bristol stool scale. Type 1 and 2 were grouped into Constipation. Type 3, 4 and 5 were grouped into Normal. Type 6 and 7 were grouped into Diarrhea. Illustration from (Chumpitazi et al., 2016; Lewis & Heaton, 1997)

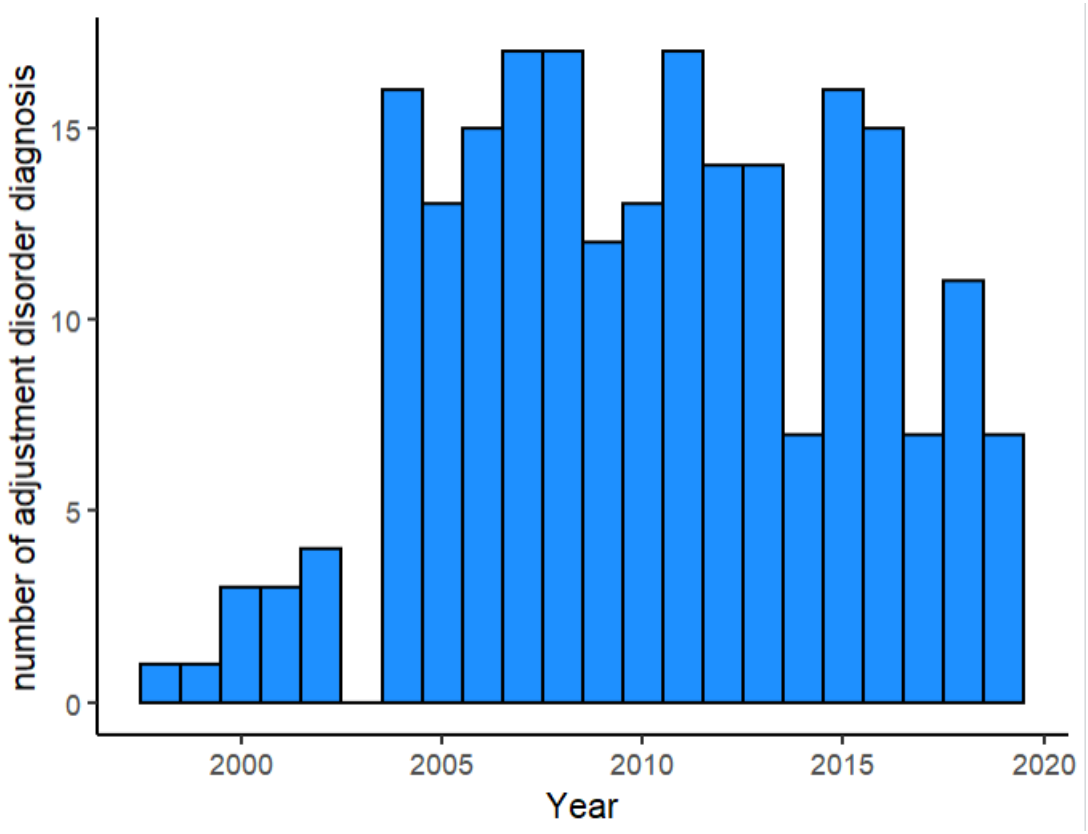

Figure 2. The number of AD diagnosis, only counting the most recent diagnosis from each participant.

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