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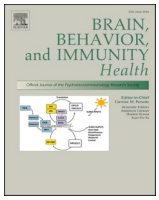

Disrupted gut harmony in attention-deficit/hyperactivity disorder: Dysbiosis and decreased short-chain fatty acids

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ABSTRACT

Background: Attention-Deficit Hyperactivity Disorder (ADHD) is a prevalent neurodevelopmental disorder with complex genetic and environmental underpinnings. Emerging evidence suggests a significant role of gut microbiota in ADHD pathophysiology. This study investigates variations in gut microbiota composition and Short-Chain Fatty Acid (SCFA) profiles between children and adolescents with ADHD and healthy controls.
Methods: The study included 42 ADHD patients and 31 healthy controls, aged 6–18 years. Fecal samples were analyzed for microbial composition using 16S rRNA gene sequencing and for SCFA profiles through gas chromatography-mass spectrometry (GC-MS). The study assessed both α and β diversity of gut microbiota and quantified various SCFAs to compare between the groups.
Results: ADHD subjects demonstrated significantly reduced gut microbiota diversity, as indicated by lower α-diversity indices (Shannon index, Observed species, Faith PD index) and a trend towards significance in β-diversity (Weighted UniFrac). Notably, the ADHD group exhibited significantly lower levels of key SCFAs, including acetic, propionic, isobutyric, isovaleric, and valeric acids, highlighting a distinct microbial and metabolic profile in these individuals.
Conclusion: This study uncovers significant alterations in gut microbiota and SCFA profiles in children with ADHD, compared to healthy controls. The observed changes in SCFAs, known for their associations with other behavioral and neurologic pathologies, and for their role in neural signaling. These findings offer a metabolite fingerprint that could potentially lead to novel diagnostic and treatment approaches for ADHD, emphasizing the importance of gut microbiota in the disorder's pathogenesis and management.

1. Introduction

Attention-Deficit Hyperactivity Disorder (ADHD) constitutes the most common neurodevelopmental disorder in children and often persists into adulthood. ADHD affects 5.3–7.2% of children and is associated with various adverse outcomes, including educational underachievement, employment difficulties, and increased risk of other mental health disorders (American Psychiatric Association 2013; Thomas et al., 2015). Approximately 30–60% of children with ADHD continue to show symptoms into adulthood, resulting in 1–6% of the adult population having ADHD (Wender et al., 2001). Early diagnosis and treatment can improve long-term outcomes (Sayal et al., 2018).

ADHD arises from various factors, including genetic predisposition and environmental influences, which are often related to lifestyle. It is characterized by high levels of impulsivity, hyperactivity, and inattention, which can interfere with normal functioning (Atkinson and Hollis 2010). These behaviors described as premature and thoughtless actions (impulsivity), restless and excessive movement (hyperactivity), and a disorganized style preventing sustained effort (inattention) (Atkinson and Hollis 2010). The disorder shows positive correlations with other neuropsychiatric disorders and various clinical conditions such as allergies.

Over the past several years, a growing body of research has underscored the vital role played by the gut microbiota in human health, particularly in relation to neuropsychiatric disorders (Bull-Larsen and Hasan Mohajeri 2019; Checa-ros et al., 2021). The gut microbiota,

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comprising up to 100 trillion microorganisms in the gastrointestinal tract, plays a crucial role in human health by breaking down nutrients, producing vitamins, protecting against pathogens, and regulating the immune system (Cénit et al., 2014; Koh et al., 2016). Gut microbiota has been recognized to influence brain function and behaviors through a bidirectional communication system known as the microbiota-gut-brain axis (MGBA) (R. M. Stilling et al. 2014). This axis influences brain function and behavior through neural, immune, and endocrine pathways (Cryan and Dinan 2012; Martin et al., 2018; Mohajeri et al., 2018).

Numerous studies have indicated that alterations in microbial abundance, richness, and diversity are associated with a variety of neuropsychiatric conditions, including but not limited to ADHD, anxiety, depression, and Autism Spectrum Disorder (ASD) (Koh et al., 2016; Roman M. Stilling et al., 2016). Research has shown that ADHD patients often exhibit lower gut microbiota diversity compared to healthy controls, suggesting a link between reduced microbial diversity and the disorder (Chong et al., 2020; Dhariwal et al., 2017; Gagliardi et al., 2018; Prehn-Kristensen et al., 2018). Additionally, β-diversity analysis has shown potential variations in gut microbiota composition between ADHD and control groups (Jiang et al., 2018; Wang et al., 2020). These findings highlight the significance of the MGBA and the interplay between the gut microbiota and the Central Nervous System (CNS), suggesting that the microbiome may offer novel targets for the development of interventions to manage and treat neuropsychiatric disorders (Bull--Larsen and Hasan Mohajeri 2019; Checa-ros et al., 2021).

One potential mechanism through which the gut microbiota impacts human health involves the production of metabolites, which can be either harmful or beneficial. Among these, specific gut bacteria are capable of fermenting dietary fiber in the colon, leading to the production of Short-Chain Fatty Acids (SCFAs). Approximately 95% of gut SCFAs are produced as a byproduct of microbial fermentation of undigested carbohydrates. SCFAs, including acetate, propionate, and butyrate, play a crucial role in various physiological processes, including maintaining gut health, modulating the immune system, and influencing brain function (Flint et al., 2002; Koh et al., 2016; Reed 2012; Ríos-Covián et al., 2016).

For example, butyrate has anti-inflammatory effects and may prevent colon cancer, while propionate can protect the blood-brain barrier and stimulate the secretion of norepinephrine (Erny et al., 2015; Hoyles et al., 2018). Additionally, SCFAs have been shown to influence neurotransmitter production, particularly serotonin (5-HT) and norepinephrine (NE), which are critical in ADHD pathophysiology (Fukumoto et al., 2003; Inoue et al., 2012; Reigstad et al., 2015; Yano et al., 2015).

This study aims to uncover potential differences in gut bacterial profiles and SCFA levels between children with ADHD and healthy peers, focusing on those aged 6–18. This approach, a first in this specific age group, not only sheds light on the gut microbiota's metabolic activity in ADHD but also sets a foundation for future research into gut-brain connections in neuropsychiatric disorders.

2. Methods and material

The ADHD patient group consisted of individuals receiving treatment at the Center for Diagnosis and Therapy of ADHD in Gdańsk, Poland. ADHD diagnosis was determined according to the diagnostic criteria of DSM-V (American Psychiatric Association 2013). Inclusion criteria for the ADHD group were continuous care at the center and an age between 6 and 18 years. The control group comprised healthy peer relatives of patients, aged 6–18 years, without any known diseases and not receiving any drugs. Exclusion criteria for both groups included severe general conditions, chronic diseases, or antibiotic treatment within three months prior to inclusion.

Participants were recruited by the staff at the Center for Diagnosis and Therapy of ADHD, who directly approached the parents of potential participants to provide detailed information about the study. Written informed consent was obtained from the parents or guardians of all participants. The study was approved by the Ethics Committee of the Poznan University of Medical Sciences, Poland (No. 1100/18 with amendment 88/19) and conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

2.1. Study population

Seventy-three Polish children (6–18 years old) participated in this study, 42 diagnosed ADHD children and 31 healthy controls as detailed in Table 1.

Twelve participants from the ADHD group reported medication use. Two participants of those ADHD medication users reported the use of allergic medications. None of subjects from healthy subjects used any medication, although one female control group participant reported having Atopic Dermatitis (see Supplementary Table S1).

2.2. Data collection

Fresh feces samples were collected from all studied subjects, refrigerated, and kept in ice packs until stored at −80 °C. Later, the samples were packed with dry ice (carbon dioxide), shipped to Israel, and were stored immediately at −80 °C before further processing. Samples were thawed once, split into five polypropylene tubes, and then re-frozen until each analysis. Studies found that SCFAs remained stable after two freeze-thaw cycles, indicating no significant degradation that would affect the analysis quality (Gu et al., 2021; Mahdi et al., 2024).

2.3. Microbiome composition analysis

Microbial DNA was extracted from fecal samples using the Dneasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA concentration and quality were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For library preparation, the V3–V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 785R primers with Illumina overhang adapter sequences, as previously reported. Amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), indexed with limited-cycle PCR using Nextera technology, and further cleaned up. The final libraries were sequenced on an Illumina MiSeq platform, with a 2 × 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing reads are available from the corresponding author upon reasonable request.

Analysis of microbiota composition utilized QIIME2 software, identifying bacteria at various taxonomy levels. A custom script in RStudio calculated the relative frequency of each taxonomic level, assigning unknown taxa to the nearest known taxonomic rank.

2.4. Short-chain fatty acids analysis

Each fresh fecal sample was thawed, weighed, and suspended (250 mg) in 1 ml of sterile acidic distilled water (with 85% ortho-phosphoric

Table 1
Study population.

	ADHD (n = 42)	Control (n = 31)
Female n (%)	6 (14.3%)	17 (54.8%)
Male n (%)	36 (85.7%)	14 (45.2%)
Age (years) Median (1st: 3rd quartile)	11.0 (8.5:11.8)	10.0 (8.0:12.0)
Normal BMI percentile[a] n (%)	32 (76.2%)	22 (71.0%)
BMI percentile Median (1st: 3rd quartile)	62 (24.5:86.8)	61 (17.8:76.8)

[a] Equal to or greater than the 5th percentile and less than the 85th according to Centers for Disease Control and Prevention (CDC, 2022).

acid at a 1:50 ratio), and the pH was adjusted to 2–3. The suspension was incubated at room temperature for 10 min with occasional shaking, followed by 2 min of vortexing. The mixture was then transferred into an Eppendorf and centrifuged for 10 min at 4 °C and 14,000 rpm. The liquid phase was then transferred into a filtered tube and centrifuged again. 225 µl of the filtrate was collected into a new Eppendorf. To this filtrate, 25 µl of 2-ethylbutyric acid solution (IS), was added. The resulting 250 µl mixture has transferred into autosampler's vials equipped with 8 mm inserts and stored in a −20 °C until analysis. The analytical system used was an Agilent 6890A coupled with an Agilent 5973C mass selective detector. The column utilized for the analysis was a DB-FFAP 122–3232, with dimensions of 0.25 mm × 30m x 0.25 µm. Helium served as the carrier gas, with a flow rate of 13.6 mL/min. The oven temperature was programmed to start at 70 °C, increase to 100 °C at a rate of 20 °C/min, then to 180 °C at 8 °C/min and hold for 3 min, and finally reach 200 °C at 20 °C/min. The injector used had a glass liner with a glass wool plug, and the sample volume for each analysis was 1 µl. The total runtime of the analysis was 15.5 min. The detector operated in Selected Ionization Mode (SIM), and SCFA identification was based on the retention time of standards (WSFA-4, Sigma-Aldrich) and reference to NIST 08 and Wiley7N libraries.

2.4.1. Comparative analysis for identifying gut microbiota biomarkers between study groups

Data on the relative prevalence of each taxonomic level were uploaded to the MicrobiomeAnalyst online platform in order to find differences in gut bacterial composition between study groups (Chong et al., 2020). This platform uses Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (Segata et al., 2011), a multidimensional algorithm for characterizing and identifying genomic biomarkers, such as genes, pathways, or taxonomic groups, in at least two biologically distinct groups. LEfSe, a nonparametric statistical approach, identifies microbial taxa with significant prevalence differences between groups. Initially, a Kruskal-Wallis sum-rank test detects significant bacterial prevalence variations. Subsequently, LDA quantifies the effect size of these differences. Consistent with scientific norms, only bacteria with an LDA score greater than 2.0 were considered significant in the analysis.

Statistical analysis to examine differences in short-chain fatty acids levels between study groups and correlations with other parameters.

The SCFAs values obtained indicate nonparametric data, and therefore a Mann-Whitney *U* test was conducted to examine differences in SCFAs levels between study groups.

The Mann-Whitney *U* test and the graphs showing the median concentration (ppm) of SCFAs were performed using a custom script in

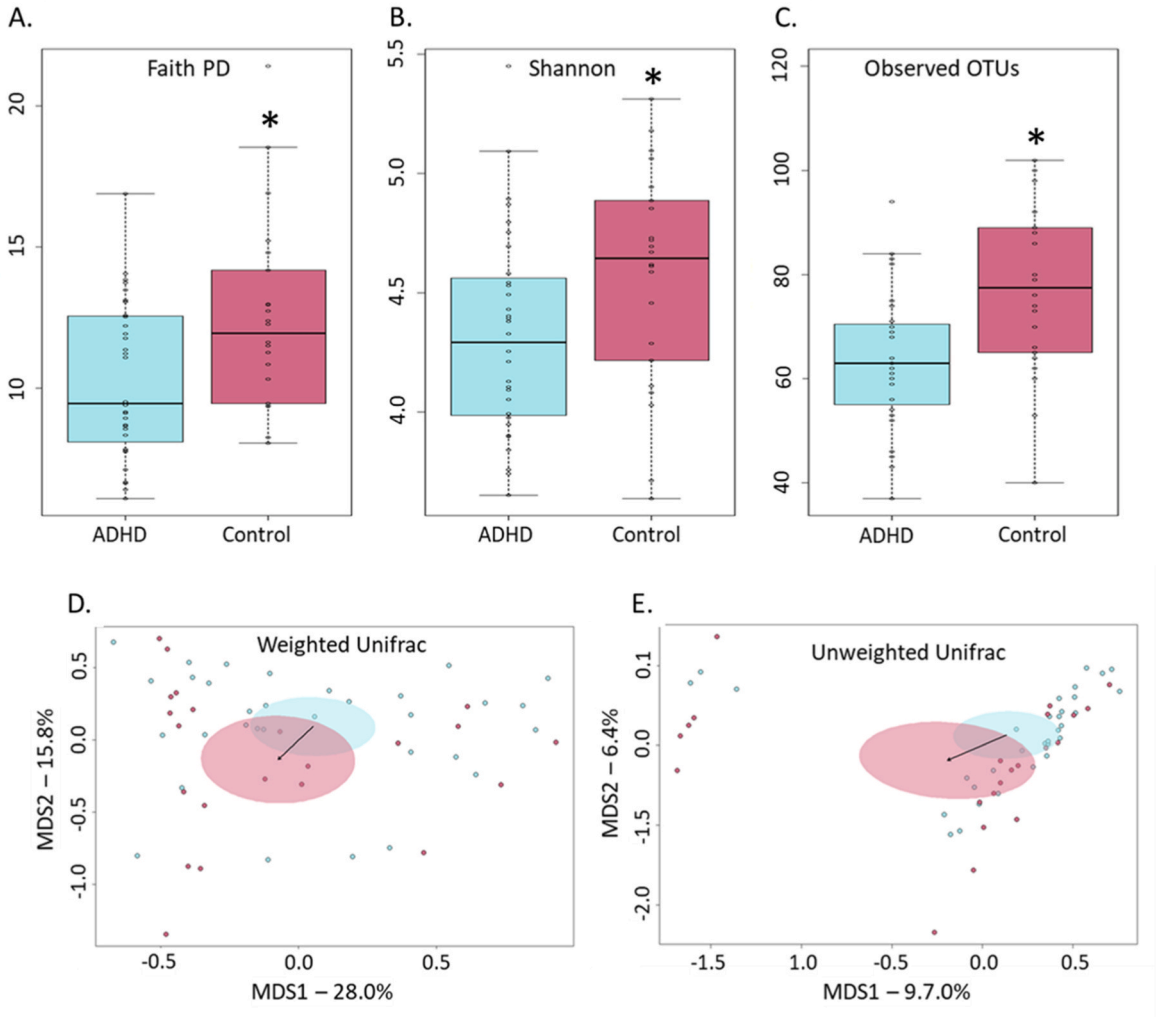

Fig. 1. *The microbiome α and β diversity of the fecal samples from the study groups.* α diversity as measured by the Faith-PD (A), Shannon diversity index (B), and Observed species index (C) for the ADHD group (light blue; n = 42) and the control group (red; n = 31). Statistical tests were performed using the Mann–Whitney test, and significance is indicated by * ($P < 0.05$). Multiple dimension scale (MDS) plots show the differences in species diversity between samples tested (β diversity). (D) Weighted Unifrac: presence/absence/abundance of OTUs (phylogenetic diversity). ANOSIM, R = 0.54, $P = 0.059$, based on 999 permutations. (E) Unweighted Unifrac: presence/absence of operational taxonomic units (OTUs) (phylogenetic richness). ANOSIM, R = 0.045, $P = 0.112$, based on 999 permutations.

RStudio (2022.02.0 + 443) (R Core Team, 2019), using the "ggplot2", "sqldf" and "gridExtra" packages (versions 3.3.5, 0.4–11, respectively).

3. Results

3.1. Attention-deficit hyperactivity disorder associated with altered α and β bacterial diversity

Variations were observed in the α diversity indices between the ADHD group and the control group (Fig. 1A–C). A noticeable decline was observed in the Shannon index ($P < 0.05$), the Observed species ($P < 0.01$), and the Faith-PD index ($P < 0.05$) in the ADHD group compared to the control group. This consistent trend across all three indices highlights the distinct bacterial diversity characterizing each group.

Differences in species diversity between samples were subsequently examined using β diversity analyses, including 1. weighted UniFrac and 2. unweighted UniFrac, as shown in Fig. 1. The Weighted UniFrac algorithm found trending towards significance difference ($P = 0.059$) in the composition of the gut bacterial population between the subjects in the ADHD group and the subjects in the Control group (Fig. 1D). Although the Unweighted UniFrac algorithm (Fig. 1E) did not indicate significant differences ($P = 0.112$) in the β range between the study groups, likely due to a small number of abnormal samples as depicted on the left in the graph, all other indices suggest a distinct microbiome profile when comparing the two groups.

Linear discriminant analysis effect size analysis reveals significant differences in bacterial prevalence between attention-deficit hyperactivity disorder and control groups at phylum and genus levels.

Identifying specific bacteria with significant variance in prevalence between the study groups was assessed at both the phylum and genus levels, representing differing levels of taxonomic resolution in the microbial community structure. However, since any unknown taxonomic level (e.g., unknown genus) was assigned to the next highest known taxonomic rank, results show additionally more taxonomic levels. This analysis revealed that the prevalence of one phylum, three familiae and three genera were significantly different ($P < 0.05$) between the study groups and had an effect size (LDA score) greater than 2 (Fig. 2). In the ADHD group, the prevalence of *Blautia* (genus) and *Lachnospiraceae* (family) were found to be significantly higher (LDA>2, $P < 0.05$). On the other hand, the prevalence of *Verrucomicrobia* (phylum) and *Akkermansia, Anaerococcus* (genera), *Christensenellaceae, and Ruminococcaceae* (familiae), were found to be significantly higher (LDA> 2, $P < 0.05$) in the control group. Three more bacteria were found trending towards significant difference (P values mentioned in Fig. 2). The genus *Anaerostipes* was higher in the ADHD group and *Clostridiales* (family) and *Tenericutes* (phylum) in the Control group. It was also found that the *Verrucomicrobia* phylum has the highest LDA score (LDA score = 5.44), the same score as the *Akkermansia* (genus), which also belongs to *Verrucomicrobia* phylum. Taxonomic analysis summery shown in Table S2.

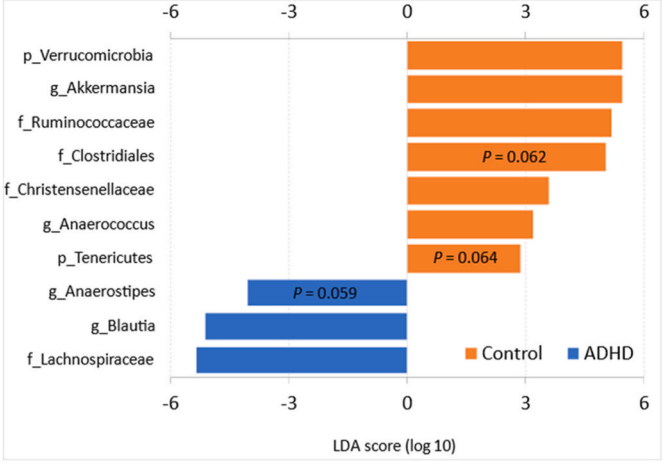

Fig. 2. *Characterization of the microbiome of the subjects from the ADHD and Control groups by LEfSe analysis.* The histogram shows a phylum and genus with LDA scores> 2 that are significantly more prevalent ($P < 0.05$) in the ADHD group (light blue; n = 42) and in the Control group (red; n = 31), as evidenced by LEfSe analysis.

3.2. Low levels of short chain fatty acids in feces samples of the attention-deficit hyperactivity disorder group

An analysis of the SCFA profiles across all study participants (n = 73) indicated that acetic acid was the predominant SCFA, with a median (Q1:Q3) concentration of 3571 (1569:8346) ppm in dry feces. This was followed by butyric and propionic acids, which recorded median concentrations of 2973 (2086:5326) ppm and 1210 (770:2247) ppm, respectively. The concentrations of isobutyric, isovaleric, and valeric acids were observed to be lower, registering average concentrations of 583 (353:763) ppm, 613 (360:980) ppm, and 723 (464:1032) ppm, respectively. Comparing the SCFA levels between the two study groups revealed a significant decline in the ADHD group (n = 42) in terms of acetic ($P < 0.01$), propionic ($P < 0.001$), isobutyric ($P < 0.01$), isovaleric ($P < 0.001$), and valeric ($P < 0.05$) acids when compared to the control group (n = 31), as delineated in Fig. 3. It is noteworthy that the concentration of butyric acid did not show a significant discrepancy between the ADHD and control groups ($P = 0.235$).

4. Discussion

In this study, we found significant differences in the gut microbiota composition and SCFA profiles between children with ADHD and healthy controls. Specifically, ADHD subjects exhibited reduced α diversity and lower levels of key SCFAs such as acetic, propionic, isobutyric, isovaleric, and valeric acids. These alterations in gut microbiota and SCFA levels suggest a distinct microbial and metabolic profile in ADHD individuals. Given the known roles of SCFAs in regulating immune function, neurotransmitter production, and maintaining gut-brain axis integrity, our findings suggest that disruptions in SCFA production may contribute to the neurodevelopmental and behavioral characteristics observed in ADHD. This study highlights the importance of gut microbiota and its metabolites in ADHD pathogenesis and suggests potential avenues for therapeutic intervention targeting gut microbial composition and SCFA production.

4.1. The microbiome profile of the study groups

α diversity in the fecal samples of subjects from both the ADHD and Control groups was analyzed using the Shannon index, Observed, and Faith-PD index. Our results showed that α diversity was significantly lower in the ADHD group compared to the Control group across all three indices.

Previous research assessing α diversity in ADHD has demonstrated inconsistent results. A German study involving 14 male ADHD patients (aged 11.9 years) and 17 controls (aged 13.1 years) reported decreased α diversity in ADHD patients, along with differences in β diversity (Prehn-Kristensen et al., 2018). Conversely, a recent study observed increased α diversity in ADHD patients according to Shannon and Chao1 indices, but a decrease with the Simpson index, which is significant as it reflects species proportion in a sample. Other studies on α diversity in ADHD have yielded mixed results, showing either increases or no significant differences compared to controls. These studies often involve varied participant profiles, including different ages, single-gender dominance, and treatment-naïve individuals, factors that can influence microbiota composition and affect the validity of direct comparisons. The strength of this study lies in its representative sample, which includes a diverse range of ages, genders, and medication uses, making it

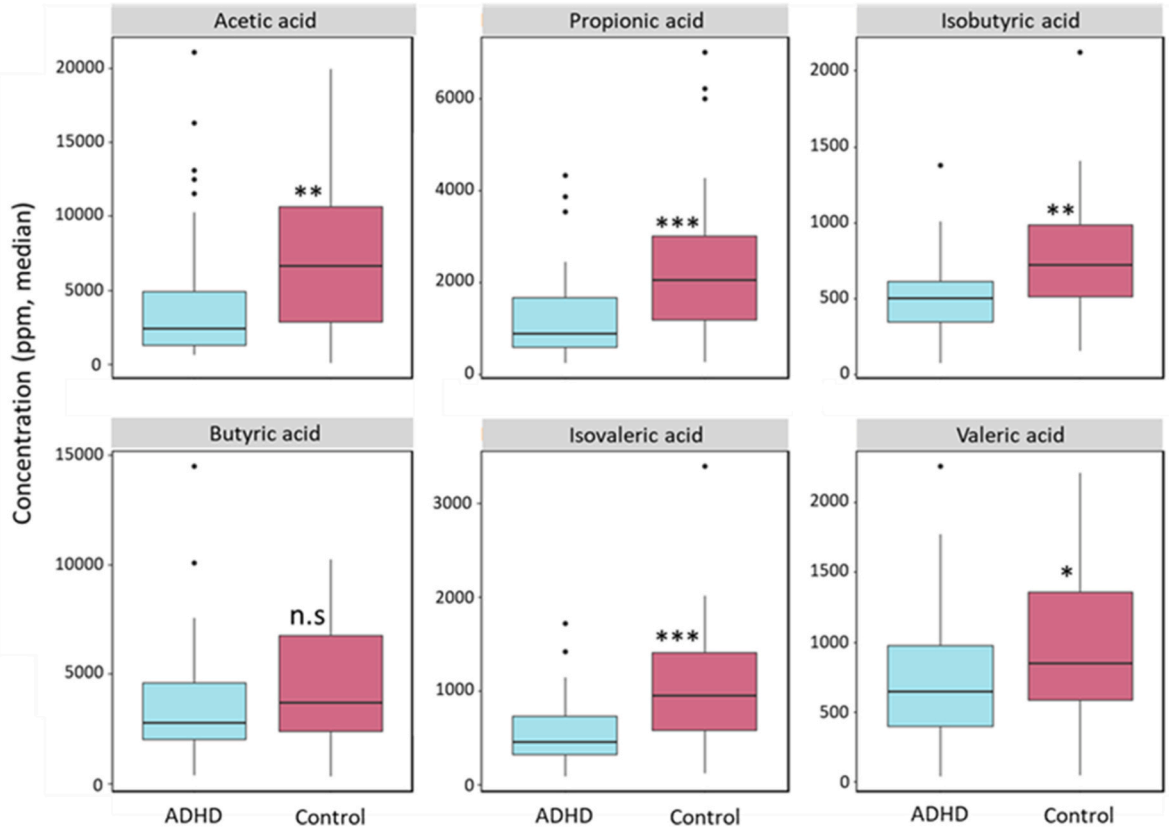

Fig. 3. *Fecal SCFA* in ADHD (light blue; n = 42) and control (red; n = 31) children (6–18 years old) representing by median. Statistical tests were performed using the Mann–Whitney test, and the significance marked *, **, and *** ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

highly reflective of the target population. As noted, α diversity encompasses both species number and their abundance distribution. Research suggests that high gut microbiota diversity inhibits pathogenic bacteria growth, thus preventing dysbiosis (Chong et al., 2020; Dhariwal et al., 2017; Gagliardi et al., 2018). Accordingly, our study's findings, indicating lower α diversity in ADHD, align with the hypothesis linking neuropsychiatric disorders like ADHD to dysbiosis markers.

β diversity analysis, employing weighted and unweighted UniFrac algorithms, revealed potential variations between ADHD and healthy control groups. Weighted UniFrac indicated a trend towards significance ($P = 0.059$) in gut microbiota composition, whereas Unweighted UniFrac did not show significant differences ($P = 0.112$). This discrepancy may stem from the limited number of abnormal samples (Fig. 1E).

Although previous studies in this field have shown slight and inconsistent differences in both α and β diversity, they consistently report significant differences at the taxonomic level, with several phyla and genera appearing repeatedly. For example, Wang et al. studied gut bacteria in 30 ADHD children (aged 8.4 ± 1.7 years, 77% male) and 30 healthy controls (aged 9.3 ± 2.2 years, 60% male), employing Unweighted UniFrac and Weighted UniFrac algorithms. Their findings indicated comparable overall gut microbiomes in both groups. However, detailed taxonomic analysis revealed differences, with five out of nine identified bacterial phyla constituting 99% of all gut bacteria (Wang et al., 2020). In a 10-week pilot involving 17 ADHD children aged 7–12, micronutrient treatment's effect on gut microbiota β diversity was examined. The participants were split into a treatment group (10 children) and a placebo group (7 children), with results showing no significant β diversity alterations post-treatment. This suggests ADHD children's gut microbiota might resist short-term environmental shifts, potentially reflecting a brain-mediated stability in intestinal microbiota. It's important to recognize, though, that gut microbiota composition is typically more affected by dietary fiber than micronutrients (Stevens et al., 2020). Another study found minor variations in β diversity between ADHD participants (n = 42) and healthy controls (n = 50), aged 13–29 years, using the betadisper algorithm, although this was not reflected in the Weighted UniFrac analysis. The study also revealed the predominance of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* phyla. At the genus level, ADHD individuals showed a reduced abundance of *Prevotella_9* (*Bacteroidota/Bacteroidia*) and *Coprococcus_2* (*Firmicutes/Clostridia*), but an increased abundance of *Intestinibacter* (*Firmicutes/Clostridia*) compared to controls (Szopinska--Tokov et al., 2020). In a Chinese study involving 51 treatment-naïve ADHD participants and 32 healthy controls, aged 6–10 years, β diversity analyses (unweighted and weighted UniFrac distances, Bray–Curtis PCoA) did not significantly distinguish the ADHD group, likely due to high individual variability. However, at the family level, differences were observed, with the ADHD group showing a decreased abundance of *Faecalibacterium*, *Lachnoclostridium* (*Firmicutes/Clostridia*), and *Dialister* (*Firmicutes/Negativicutes*) (Jiang et al., 2018).

These variations in gut microbiota composition may arise from differences in study populations, dietary habits, age, and treatment methods. Notably, Western diet is typically high in fat and calories, while Chinese diets are generally lower in fat, sugar, and meat. Such dietary differences could significantly impact gut microbiota compositions. In the current study, tendencies towards significant differences in gut microbiota were observed using the weighted UniFrac algorithm, but not with the unweighted version. These algorithms calculate distances between organisms in samples differently. Specifically, the unweighted UniFrac emphasizes rarer taxa within the population, unlike the weighted UniFrac (Fukuyama 2019; Lozupone et al., 2007). Consequently, the observed findings suggest that the differences in gut bacterial composition between ADHD and control groups likely stem from more prevalent common taxa in the subjects' gut bacterial populations.

LEfSe analysis was conducted to confirm the observed differences in

β diversity. This analysis identified seven biomarkers at the phylum, family, and genus levels that significantly differ between the study groups, with three additional biomarkers showing a tendency towards significance. Additionally, while some of the taxa identified in this analysis are familiar markers in ADHD research, others may represent less explored areas in the context of ADHD. Therefore, the discussion will primarily focus on these known markers. Seven out of ten markers capable of differentiating children diagnosed with ADHD from healthy controls are part of the *Firmicutes* phylum, specifically within the *Clostridial* order. Notably, the family *Lachnospiraceae*, within this order, showed a pronounced presence of markers, which were found at higher levels in the ADHD group. In contrast, two other families (*Christensenellaceae* and *Ruminococcaceae*) and one genus (*Anaerococcus*), also within the *Firmicutes* phylum, were observed at lower levels in the ADHD group.

Lachnospiraceae family members, particularly the genera *Blautia* and *Anaerostipes*, were found to be increased in abundance in ADHD subjects in the present study. This finding contrasts with the reduced levels of *Lachnoclostridium*, a genus within the *Lachnospiraceae* family, as reported by Jiang et al. While the literature presents inconsistent results regarding ADHD and its association with specific bacterial families and genera, it is worth mentioning that *Lachnoclostridium* has been correlated with various neurodevelopmental and neurodegenerative disorders, including ASD, Multiple Sclerosis, and Amyotrophic Lateral Sclerosis (J. Chen et al., 2016; Fang et al., 2016; Luna et al., 2017). Additionally, our study aligns with others that have found a higher abundance of *Blautia* and *Anaerostipes* in disorders such as major depressive disorder, schizophrenia, binge eating disorders (BED), and anorexia nervosa (Barandouzi et al., 2020; Leyrolle et al., 2021; Li et al., 2020; Schulz et al., 2021; Suganya and Koo 2020). Therefore, it is suggested that elevated levels of specific genera within the *Lachnospiraceae* family, particularly *Blautia* and *Anaerostipes*, may serve as potential biomarkers for ADHD.

Ruminococcaceae (also known as *Oscillospiraceae*) has been associated with less severe symptoms in schizophrenia patients compared to non-psychiatric matched subjects (Nguyen et al., 2019). A similar pattern was observed in a study contrasting neurotypical children (n = 20, aged 2.5–18 years) with autistic subjects (n = 30, age and sex-matched), revealing a distinct association with *Ruminococcaceae* (Hill-Burns et al., 2017; Liu et al., 2019). Complementing these human studies, research in mammalian models has linked an increase in *Ruminococcaceae* to the alleviation of impulsivity, attention deficits, reward-learning, and locomotor activity in novel environments. Interestingly, these behavioral changes correlated with dopamine (DA) receptor expression in the striatum (Jadhav et al., 2018; Peterson et al., 2020), suggesting a potential mechanistic pathway involving the MGBA. In cases of alcohol dependency, a psychiatric condition characterized by high intestinal permeability, a notable decrease in various *Ruminococcaceae* genera was observed, contrasted by an increase in the genus *Dorea* within the *Lachnospiraceae* family. These observations underscore the gut barrier's significance in behavioral disorders and highlight *Ruminococcaceae*'s critical role in brain health (Sophie et al., 2014).

In ADHD-Inattentive (ADHD-I) subtype patients, there was a significant decrease in the *Ruminococcaceae* family at the family level. Conversely, while no overall difference in *Ruminococcaceae* abundance was noted between ADHD-H (hyperactive-impulsive subtype) and control subjects, a decrease in the genus *Faecalibacterium* within the *Ruminococcaceae* family, was observed in the ADHD-H subtype. This reduction in *Faecalibacterium* aligns with findings from other ADHD studies, further supporting its potential role in ADHD symptomatology (Jiang et al., 2018; Prehn-Kristensen et al., 2018).

Verrucomicrobia, one of the six most abundant phyla in the human gut, was notably highlighted in our LEfSe analysis, having the highest LDA score (−5.44 log10), a distinction also shared by the *Akkermansia* genus within this phylum. This suggests a reduced presence of these bacteria in ADHD subjects. Echoing our findings, Fan et al. (2019) reported similar observations in a study involving various ADHD subtypes (ADHD-Inattentive n = 21, ADHD-Combined n = 20, ADHD-hyperactive-impulsive n = 18) compared to 23 healthy controls (Fan et al., 2019). In murine models, *Akkermansia* from the *Verrucomicrobia* phylum has been linked to inhibiting pathological brain changes and improving spatial learning and memory in Alzheimer's disease (AD) models, as well as reversing impaired spatial working memory with specific probiotic treatments (Higarza et al., 2021; Ou et al., 2020). Notably, animal-like serotonin (5-HT) synthesis capabilities, potentially contributing to neurotransmitter production, have been identified in nearly 20% of gut-associated genomes, including the *Akkermansia* genus (Tsavkelova et al., 2006; Valles-Colomer et al., 2019). Furthermore, *Verrucomicrobia* has been associated with protection against cognitive impairment caused by chemotherapy, showing significant correlations with attention, executive function, and memory in neurologically healthy older adults (Manderino et al., 2017; Subramaniam et al., 2020). The reduction of *Akkermansia* is also observed in other neurological disorders like BED and ASD (Iglesias-Vázquez et al., 2020; Leyrolle et al., 2021). These findings contribute to the growing evidence that *Verrucomicrobia*, especially *Akkermansia*, may significantly influence learning, memory, and attention processes.

Tenericutes has been associated in both murine models and human studies with various neurodegenerative disorders, including Parkinson's Disease, AD, ASD, anxiety, and insomnia, demonstrating both positive and negative connotations. (Y. Chen et al., 2019; Nagu et al., 2021; Nakazawa-Miklasevica et al., 2021; Syeda et al., 2018; T. Zhang et al., 2022). In this context, the present study revealed that the healthy control group tended to have greater abundances of *Tenericutes* ($P = 0.064$).

While there is growing evidence supporting the findings above, it's important to note that some studies report contradictory results. This variation underscores the complexity of gut microbiome interactions and their role in neurodevelopmental disorders. In future studies, it will be crucial to reduce potential biases and enhance data collection. This includes gathering detailed dietary information through food frequency questionnaires (FFQ), closely assessing the severity of ADHD, and incorporating serum collection. These steps will be key in accurately characterizing biomarkers that can distinguish between ADHD and healthy control subjects.

4.2. SCFA analysis

In the present study, total SCFAs concentration was significantly higher in the healthy control group. All assessed SCFAs were higher in the control group, except for butyrate, which did not show a statistically significant difference. As mentioned above, the *Firmicutes* and *Verrucomicrobia* phyla were distinguished between the study groups. Within *Firmicutes*, the *Eubacteriales* order, which includes the *Ruminococcaceae* and *Lachnospiraceae* families, is known for butyrate production (Louis and Flint 2009, 2017). Our results indicate that while these two families were abundantly found, each was predominantly present in a different study group. The *Lachnospiraceae* family was found more abundant in ADHD patients, with *Blautia* and *Anaerostipes* being the most prominent genera. Conversely, the *Ruminococcaceae* family was more prevalent in healthy control subjects, alongside, to a lesser extent, the *Christensenellaceae* family and *Clostridiales* order, which tend to be significant.

The *Firmicutes* phylum encompasses a diverse array of known SCFA producers (Magne et al., 2020). However, this perspective may oversimplify the capabilities for SCFA production, as not all members within a given taxa necessarily share identical metabolic capabilities, and certainly not all SCFA-producing taxa have been characterized to date. A notable discovery in our study is the differential abundance of two distinct families within the *Firmicutes* phylum, typically associated with butyrate production, in separate study groups. This observation could potentially account for the non-significant difference in butyrate levels between the groups. However, several potential sources of bias, such as intestinal transit and permeability, metabolite transportation, and

sample handling, should be considered (Primec et al. 2017). Illustrating this complexity, lactate-utilizing *Eubacterium hallii* and *Anaerostipes hadrus* (*Lachnospiraceae*) demonstrated decreased butyrate production (65% and 96% less, respectively) when co-cultured with *Desulfovibrio piger* (*Proteobacteria*), due to lactate competition with this Sulfate-Reducing Bacteria and *E. hallii* (Marquet et al., 2009).

As previously described, the *Verrucomicrobia* phylum and specifically the genus *Akkermansia*, were found to be abundant in the healthy control group. *Akkermansia muciniphila,* a mucin-degrading bacterium within the *Akkermansia* genus*,* is considered a producer of both propionate and acetate, mainly through the succinate pathway (Derrien et al., 2004; Louis and Flint 2017). The above results align with studies in animals and humans, showing a positive correlation between gut-microbiota dysbiosis, low SCFA levels, and various neurological pathologies, including depression, Parkinson's Disease, AD, ASD and ADHD (Borre et al., 2014; Dalile et al., 2019; Deng et al., 2019; Fan et al., 2019; Sharon et al., 2019; L. Zhang et al., 2017).

The influence of SCFAs on ADHD may also involve the regulation of neurotransmitters, notably 5-HT, predominantly produced in the gut. Approximately 90% of 5-HT is synthesized in the intestinal epithelium by Enterochromaffin cells (ECCs), influenced by metabolites from gut bacteria (Jenkins et al., 2016). SCFAs enhance 5-HT production in ECCs by upregulating Tryptophan Hydroxylase-1 (TPH1), the key enzyme in 5-HT biosynthesis (Fukumoto et al., 2003; Reigstad et al., 2015; R. M. Stilling et al. 2014; Yano et al., 2015). The secreted 5-HT, both into the colon lumen and towards the mucosa, may impact brain functions through interactions between ECC neuropods (Fig. S1) and afferent nerve fibers (Yano et al., 2015). Norepinephrine (NE), another neurotransmitter linked to ADHD, is influenced by SCFAs. Propionate, via Free-Fatty-Acid Receptors, FFAR2/3, on norepinephrinergic sympathetic nerves, promotes NE release in the CNS (Inoue et al., 2012; Kimura et al., 2011; Roman M. Stilling et al., 2016). NE (and DA) deficiencies in brain synapses are associated with ADHD symptoms, with most ADHD medications targeting these catecholamine pathways (Arnsten and Li 2005). ADHD patients typically exhibit lower NE levels, but dietary interventions that increase NE have shown symptom alleviation. This suggests a microbiota-nutrition mechanism impacting NE levels (Meguid et al., 2018).

While positive effects are frequently reported, there are studies documenting negative associations between SCFAs and negative health outcomes in certain contexts. For instance, elevated SCFA levels, particularly acetate, have been linked to neuroinflammation and microglial activation in Parkinson's Disease, contributing to motor impairment and disease progression (Erny et al., 2015). High SCFA concentrations can also influence the autonomic nervous system, enhancing sympathetic nerve activity and increasing arterial blood pressure, potentially leading to hypertension (Kimura et al., 2011; Pluznick et al., 2013). Moreover, excessive acetate production can drive parasympathetic vagal stimulation, leading to hyperinsulinemia, elevated ghrelin levels, and obesity (Perry et al., 2016). Additionally, elevated levels of SCFAs like acetate and valerate are associated with increased pro-inflammatory cytokine production and decreased anti-inflammatory cytokine levels, contributing to chronic inflammation and conditions like Alzheimer's Disease (Chong et al., 2020; Erny et al., 2015).

Although these examples highlight potential negative outcomes, instances of negative impacts are less common and may be influenced by factors such as the overall microbial composition, host genetics, diet, and specific health conditions. Differences in intestinal permeability can also affect SCFA levels. High permeability can result in increased transport of SCFAs into the bloodstream, which can impact the body. Healthy individuals may have better-regulated intestinal permeability, preventing excessive SCFA transport and maintaining their beneficial effects (Bruning et al., 2020). Higher microbial diversity in healthy subjects can lead to a more balanced production of SCFAs, contributing to overall health and reducing the risk of negative outcomes.

The current study identifies reduced microbiota diversity in ADHD subjects compared to controls, coupled with lower levels of bacterial metabolites linked to neurodevelopmental disorders. These insights enhance our understanding of the MGBA in ADHD, paving the way for further research that could lead to novel diagnostic and therapeutic strategies.

4.3. Study limitations

We suggest collecting detailed information about the type and severity of ADHD among participants. Furthermore, including data on the frequency of food intake, such as through FFQ, could provide additional insights into the gut microbiota and SCFA profiles. Addressing these aspects in future studies will enhance the understanding of the relationships between gut microbiota, SCFAs, and ADHD.

CRediT authorship contribution statement

Rafi Steckler: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Faiga Magzal:** Formal analysis, Data curation. **Marta Kokot:** Project administration, Data curation. **Jaroslaw Walkowiak:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Snait Tamir:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that there are no financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work. This includes the absence of any direct or indirect financial or personal relationships, interests, or affiliations that could be perceived as a conflict of interest relevant to the paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100829.

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