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Journal of Psychiatric Research 156 (2022) 36–43

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/jpsychires

Lower plasma concentrations of short-chain fatty acids (SCFAs) in patients with ADHD

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ARTICLE INFO

Keywords:
Propionate
Butyrate
Acetate
Succinate
Antibiotic medication
Psychostimulant medication

ABSTRACT

Short-chain fatty acids (SCFAs), produced during bacterial fermentation, have been shown to be mediators in the microbiota-gut-brain axis. This axis has been proposed to influence psychiatric symptoms seen in attention deficit hyperactivity disorder (ADHD). However, there is no report of plasma SCFA concentrations in ADHD. The aim of this study was to explore the plasma concentrations of SCFAs in children and adults with ADHD and the possible factors that could influence those levels. We collected data on age group, sex, serum vitamin D levels, delivery mode, body mass index, diet, medication and blood samples from 233 ADHD patients and 36 family-related healthy controls. The concentrations of SCFAs and the intermediary metabolite succinic acid, were measured using liquid chromatography-mass spectrometry. Adults with ADHD had lower plasma concentrations of formic, acetic, propionic and succinic acid than their healthy family members. When adjusting for SCFA-influential factors among those with ADHD, children had lower concentrations of formic, propionic and isovaleric acid than adults, and those who had more antibiotic medications during the last 2 years had lower concentrations of formic, propionic and succinic acid. When adjusting for antibiotic medication, we found that among children, those currently on stimulant medication had lower acetic and propionic acid levels, and adults with ADHD had lower formic and propionic acid concentrations than adult healthy family members. In all, our findings show lower-than-normal plasma concentrations of SCFAs in ADHD explained in-part by antibiotic medication, age and stimulant medication. Whether or not this is of clinical significance is yet to be explored.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a common neuropsychiatric disorder with a prevalence of 7.1% among children and 5% among adults, and an overrepresentation in males (Willcutt, 2012). Several studies have reported an altered gut microbiome in ADHD compared to healthy controls (Aarts et al., 2017; Cenit et al., 2017; Checa-Ros et al., 2021; Jiang et al., 2018; Richarte et al., 2018, 2021; Sukmajaya et al., 2021; Szopinska-Tokov et al., 2020; Wang et al., 2020). Although the ADHD-associated microbiome varies across these reports, findings of a difference compared to controls, together with behavioral effects observed in mice transplanted with a gut microbiome from ADHD patients (Tengeler et al., 2020), suggest an altered microbiota-gut-brain axis state in ADHD that may contribute to the pathophysiology of some behaviors in ADHD. One group of important mediators for the gut-brain axis is short-chain fatty acids (SCFAs) being carboxylic acids with less than 6 carbons. They are produced at certain levels by many bacterial species during bacterial fermentation of mainly dietary fibres in the gastro-intestinal (GI) tract (Martin-Gallausiaux et al., 2021). Succinic acid is an intermediary step in this fermentation towards propionic acid. Some SCFAs are also present in food (acetic acid, propionic acid and butyric acid). SCFAs act not only as the energy source for the local intestinal cells, but also as systemic mediators through their regulation of immune, nervous and metabolic activities

Abbreviations: ADHD, attention deficit hyperactivity disorder; SCFAs, Short-chain fatty acids.

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https://doi.org/10.1016/j.jpsychires.2022.09.042
Received 25 May 2022; Received in revised form 21 August 2022; Accepted 23 September 2022
Available online 28 September 2022

(Duscha et al., 2020; Erny et al., 2015; Hoyles et al., 2018; Yang et al., 2020a). Of the SCFAs mainly acetic, propionic and butyric acids have been investigated in health and disease (Tan et al., 2014). They regulate gene expression through inhibition of histone deacetylases, and they activate G-protein-coupled receptors (Kim and Shin, 2018; Tan et al., 2014). They can regulate key neurological activities and behavioral processes, as exemplified in the **Discussion**. However, the dose-response relationships at concentrations not far from physiological levels for the different SCFAs are yet to be determined, and importantly, the physiological levels differ a lot between the SCFAs, and between body compartments (Human Metabolome Database, http://www.hmdb.ca/). As aforementioned, the concentrations of SCFAs and succinic acid are dependent on the gut microbiome species profile and the substrate for their fermentation (diet) (Baxter et al., 2019; De Filippis et al., 2016). Moreover, diet also has a direct impact on gut microbiota composition, diversity, and richness (Makki et al., 2018). Another factor potentially with marked effects on the microbiome, and consequently on the SCFA concentrations, is antibiotics. These drugs kill or repress not only the treatment-targeted pathogenic bacteria but also the normal bacteria in gut, which can disrupt the normal composition and function of gut microbiota, such as SCFA production (Willing et al., 2011). Still, only a few studies have reported effects of antibiotic drug use on SCFA concentrations in humans (see **Supplementary Discussion**). Some gut microbiome-influencing factors have been reported to have a different distribution in ADHD compared to healthy controls, such as diet (Del-Ponte et al., 2019; Shareghfarid et al., 2020), body mass index (BMI) (Kase et al., 2021), sex, blood vitamin D levels (Kotsi et al., 2019), delivery mode (Zhang et al., 2019), and melatonin medication (Zhu et al., 2018).

To our knowledge, there is no data on the concentrations of plasma SCFA in patients with ADHD. The aims of our study were (i) to explore plasma concentrations of SCFAs in children and adults with ADHD compared to healthy individuals, and (ii) to evaluate whether age group, sex, serum vitamin D levels, delivery mode, BMI, diet and pharmacological medication including antibiotics, melatonin and stimulant medication affect SCFA concentrations in persons with ADHD.

2. Materials and methods

2.1. Participants

All participants, including ADHD patients and healthy controls, were recruited through a double-blind randomized controlled trial (ISRCTN57795429) of Synbiotic 2000 intervention performed between 2016-01 and 2018-06 at psychiatric clinics in Stockholm, Sweden as previously described (Skott et al., 2020), but only baseline data was used in this study. Patients had an ICD-10 F90 diagnosis (n = 248) and were 5–55 years old. They were on a stable pharmacological treatment (the last four weeks before recruitment), were not on antibiotic treatment (the last six weeks) and did not have any autism or GI diagnosis (except for irritable bowel syndrome), diabetes or celiac disease. In parallel, healthy individuals (n = 36) from the same family of some patients were also recruited in the same period. The controls fulfilled the criteria but had no ADHD diagnosis. Medication data collected included ADHD medication, antibiotics and melatonin. Each participant responded to questionnaires on psychiatric symptoms, GI symptoms, diet and vitamin D supplements, and non-fasting venous blood samples were collected. Finally, of the 248 ADHD patients recruited, 233 provided blood samples and were successfully assessed for plasma SCFA concentrations and included for data analysis (i.e. missing $n_{child} = 11$, $n_{adult} = 4$). The study was approved by the Stockholm Ethics Review Board, and written informed consent was obtained from all participants.

2.2. Assessment of diet

A food-frequency questionnaire, covering 4 weeks retrospectively, was used to obtain information about the participants' food intake. It was based on the ETICS diet study questionnaire and consisted of 57 items representing common food units or common food groups (Kautto et al., 2014). The answer options ranged from "2 times or more per day" to "never in the last four weeks". The energy (kcal/week) for the 57 food items was derived from the answers based on the Swedish national food agency's nutrition content database's portion sizes and nutrient compositions adjusted for age and sex (http://www7.slv.se/SokNaringsinnehall/). The data was translated from energy (kcal/week) per food item for each individual into 12 core or non-core food groups with energy percentage of the total energy intake for each individual, similar to that described in (Yap et al., 2021). Minor modifications were made regarding food items in the core/non-core food groups to capture the Swedish eating habits. The core/non-core food groups in this paper consist of 12 core (vegetables, fruits, meat, alternative proteins [egg, fish, seafood, soybean products], grain & whole grain cereals, dairy) and non-core (sweet drinks, packed snacks, sweets, take away, refined cereal, fatty meats) food groups. To use the dietary data in further analyses, principal components (PCs) from the energy percentage of the core/non-core food groups were made, called dietary PCs (Sup Fig. S1). Each individual got a coordinate of each PC (PC1, PC2 and PC3) from a principal component analysis (PCA) and they are based on percent of energy intake from 12 different food groups. PCs from a PCA on food data from the ADHD patients and healthy controls are shown in Table 1.

2.3. Serum vitamin D measurement

Serum vitamin D concentrations, being 25-OH-vitamin D, were measured using immunochemistry and electrochemiluminescence according to the normal routine at the Karolinska University Hospital Laboratory (https://www.karolinska.se/KUL/Alla-anvisningar/Anvisning/9123). The clinical reference levels of S-25-OH-Vitamin D is 50–250 nmol/L. Levels between 25 and 49 nmol/L are defined as insufficient, < 25 nmol/L as deficient, > 250 nmol/L as toxic.

2.4. Analysis of plasma SCFA concentrations

The concentrations of formic, acetic, propionic, butyric, isobutyric, succinic, valeric, isovaleric and caproic acid, were analyzed in EDTA plasma by liquid chromatography–mass spectrometry (LC-MS) according to a method described previously (Han et al., 2015) with some modifications at Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg (**Supplementary methods**). Twenty-one samples in singlets from both ADHD patients and controls were run in each batch. In total eleven 96-well batches were analyzed in two rounds (6 batches in first round in March 2020, and 5 batches in second round in July 2020). Twenty-two patient samples were analyzed in both rounds, selected to cover the range of the values in the first round. Three SCFAs (isobutyric, valeric and caproic acid) were excluded from data analysis because of the poor agreement between the 22 rerun samples analyzed in both rounds, leaving six analytes to study (Sup Fig. 2A). All plasma samples had before analysis undergone two freeze/thaw cycles. For each batch, two quality controls (QCs) for each analyte with levels in the range found in our patient samples were run in triplicates and were used to calculate the within-batch coefficient of variation being 9% (5%–11%) for the six SCFAs. The between-batch variation for the two rounds was controlled by normalizing the sample values with the same QCs in each batch. The normalization ratio for each analyte per batch was calculated as (mean of the QC values of the individual batch)/(mean of the total QC values from all batches run in the same round). All the statistical analyses for SCFA levels were performed on normalized data.

2.5. Statistical analysis

Demographic descriptive statistics were presented with median and

Table 1
Demographic and clinical characteristics of the study participants.

		ADHD (n = 233)	Family controls (n = 36)
		Median (IQR)/N (%)	Median (IQR)/N (%)
Age [years]		27 (14–38)	43 (35–44.2)
Age group	Child	88 (37.8)	0
	Adult	145 (62.2)	36 (100)
Sex	Female	131 (56.2)	19 (52.8)
	Male	102 (43.8)	17 (47.2)
Vitamin D level [nM]		70 (53–95)	58 (38–83.5)
Vitamin D category	Normal [>50 nM]	183 (78.5)	22 (61.1)
	Insufficient [≤50 nM]	47 (20.2)	13 (36.1)
	Missing	3 (1.3)	1 (2.8)
Delivery mode	Vaginal	198 (85)	32 (88.9)
	C-section	31 (13.3)	3 (8.3)
	Unknown	4 (1.7)	1 (2.8)
BMI [kg/m^2][a]		23.9 (22.2–26.9)	23.7 (22.7–27.6)
PC1[b]		−0.2 (−0.9–0.7)	0.4 (−0.4–1.2)
PC2[b]		−0.02 (−0.8–0.9)	0.3 (−0.9–1.1)
PC3[b]		0.1 (−0.7–0.8)	0.3 (−0.5–1.0)
Antibiotic use[c]	No	156 (67)	26 (72.2)
	Yes	67 (28.8)	9 (25)
	Unknown	10 (4.3)	1 (2.8)
Melatonin[d]	No	167 (71.7)	34 (94.4)
	Yes	66 (28.3)	2 (5.6)
ADHD medication[d, e]	No	75 (32.2)	36 (100)
	Yes	158 (67.8)	0 (0)
	Stimulant	150	
	Non-stimulant	13	
Season of blood sampling	Summer	90 (38.6)	7 (19.4)
	Winter	143 (61.4)	28 (77.8)

Results are given as median (25th–75th percentile [IQR]) or as number (%) of subjects.

[a] BMI only obtained from adults.

[b] PC (principal component) coordinates from PC analysis (PCA) on the food frequency questionnaire (EPIC) data with information on 57 nutrients as described in section 2.2 and Sup Fig. S1.

[c] antibiotic drug use in the last two years.

[d] ADHD medication and melatonin in the last 3 months.

[e] ADHD medications for children include the stimulants Methylphenidate, Lisdexamphetamine, the nonstimulant Atomoxetine, and for adults they include Methylphenidate, Lisdexamphetamine, Dexamphetamine, Atomoxetine. Five patients were currently on both stimulant and nonstimulant medication.

interquartile range (IQR) or count and percentage. Analysis of differences in levels of SCFA concentrations between diagnosis groups (control versus patient), between age groups (child versus adult), between sexes (female versus male), delivery modes (vaginal versus C-section), and medication groups (yes versus no) were performed using Mann–Whitney *U* tests. Associations between ADHD medication and SCFA concentrations were assessed in adults and children separately. This was because ADHD medication previously was associated with higher vascular inflammatory cytokines in children only (Yang et al., 2020b), and that some SCFAs were shown to protect against upregulation of these cytokines (Zajkowska and Mondelli, 2014). Statistical relationships for plasma SCFA concentrations to serum vitamin D levels, BMI, dietary PC1-3, and number of antibiotic doses the last 2 years, were assessed applying Spearman's rank correlation tests. To correct for multiple testing, statistical significance was set at $\alpha = 0.017$, meaning correction for 3 independent tests for the six SCFAs, as the four of the six SCFAs were highly correlated (Spearman's rank correlation > 0.4) with each other (FDR-corrected p values indicated in Sup Fig. 2B). Finally, regression analyses with backward elimination method using AIC (Akaike information criterion) criteria were conducted to fit a model for the levels of SCFAs in patients, and then in all adults. Concentrations of SCFAs and vitamin D were natural logarithm transformed to obtain a close-to normal distribution. All statistical analyses were performed using R programming language version 3.6.3. Graphs were made using ggplot 2 package from R.

3. Results

3.1. Plasma SCFA concentrations in patients with ADHD and healthy family controls

The characteristics of the 233 ADHD patients and 36 family-related controls who provided blood samples for SCFA measurements, including 88 children and 181 adults are shown in Table 1. All the family-related controls included were adults, therefore we compared the levels of SCFAs between patients and controls only among adults, using $\alpha = 0.017$ as cut-off for statistical significance. We found that ADHD patients had lower formic acid, acetic acid, propionic acid and succinic acid levels in plasma than healthy family members (Fig. 1A). Even when studying only the pairs from the same family (n = 18), we found a tendency for that ADHD patients had lower formic acid than healthy controls ($p = 0.021$, **Sup** Fig. 2C). Factors studied here, previously proposed to influence intestinal microbiome, including age group, sex, vitamin D levels, delivery mode, BMI, dietary PCs, medication with antibiotics and melatonin, as well as core ADHD medications are listed in Table 1 (Cui et al., 2021; Dominguez-Mozo et al., 2021; Holota et al., 2019; Mueller et al., 2021; Nilsen et al., 2020; Willing et al., 2011). The factors were independently tested for association with the SCFA concentrations in the ADHD patients. We found that age group (adult/child) (Fig. 1B), sex (female/male) (Fig. 1C), serum vitamin D levels (Fig. 2A), delivery mode (vaginal/C-section) (Fig. 2B), and dietary PC2 (Fig. 2C) and number of doses with antibiotic drug use the last 2 years (Fig. 2D) associated with plasma concentrations of at least one of the measured SCFAs. Adult patients had higher levels of formic, acetic, propionic and isovaleric acid than child patients (Fig. 1B). Females had higher formic and acetic acid than males (Fig. 1C). The concentrations of formic acid in ADHD patients associated positively with vitamin D levels ($p = 0.0036$, Fig. 2A). The patients delivered by C-section had lower concentrations of succinic acid ($p = 0.0011$), and this association was mainly driven by children ($p_{child} = 0.007$, $p_{adult} = 0.067$) (Fig. 2B and **Sup** Fig. S3A). Further, dietary PC2, i.e. higher proportions of dairy, fruits, grain and whole grain cereals, vegetables, and lower proportions of non-core food groups, associated with higher acetic acid concentrations ($p = 0.0051$) (Fig. 2C and **Sup** Fig. S1). While the dietary PCs did not differ between adult patients and adult healthy controls ($p > 0.13$), PC1 and PC2 were lower in pediatric ADHD patients than adult patients ($p = 0.00089$ and $p = 0.0025$, respectively, Table S1), however, there was no difference between children and adult patients in the linear relationship between levels of SCFAs and the three dietary PCs ($p > 0.05$, **Sup** Fig. S3B). Patients with more doses of antibiotic drugs during the last 2 years had lower concentrations of all SCFAs but formic acid ($p \leq 0.017$, Fig. 2D), with similar patterns among adults and children separately (**Sup** Fig. S3C). In pediatric patients, but not adults, those on stimulant medication [yes (currently or 1–3 months ago)/no (3–24 months ago or never)] had lower levels of acetic ($p = 0.010$) and propionic acid ($p = 0.0015$) (Fig. 2E). When looking at the stimulants Methylphenidate and Lisdexamphetamine and the non-stimulant Atomoxetine separately, the children on stimulants had lower levels of propionic acid (**Sup** Fig. S4A). Meanwhile, the effects were only found in those children who were currently on medication as compared to those who had never taken such medication ($p_{acetic\ acid} = 0.0018$, $p_{propionic\ acid} = 0.0025$, **Sup** Fig. S4B). However, we could not detect any difference in the levels of SCFAs by dietary PC1 (**Sup** Fig. S5A), PC3 (**Sup** Fig. S5B) or treatment with melatonin [yes/no] (**Sup** Fig. S5C). Further, BMI, which was available only for adults, was not associated with plasma SCFA levels (**Sup** Fig. S5D).

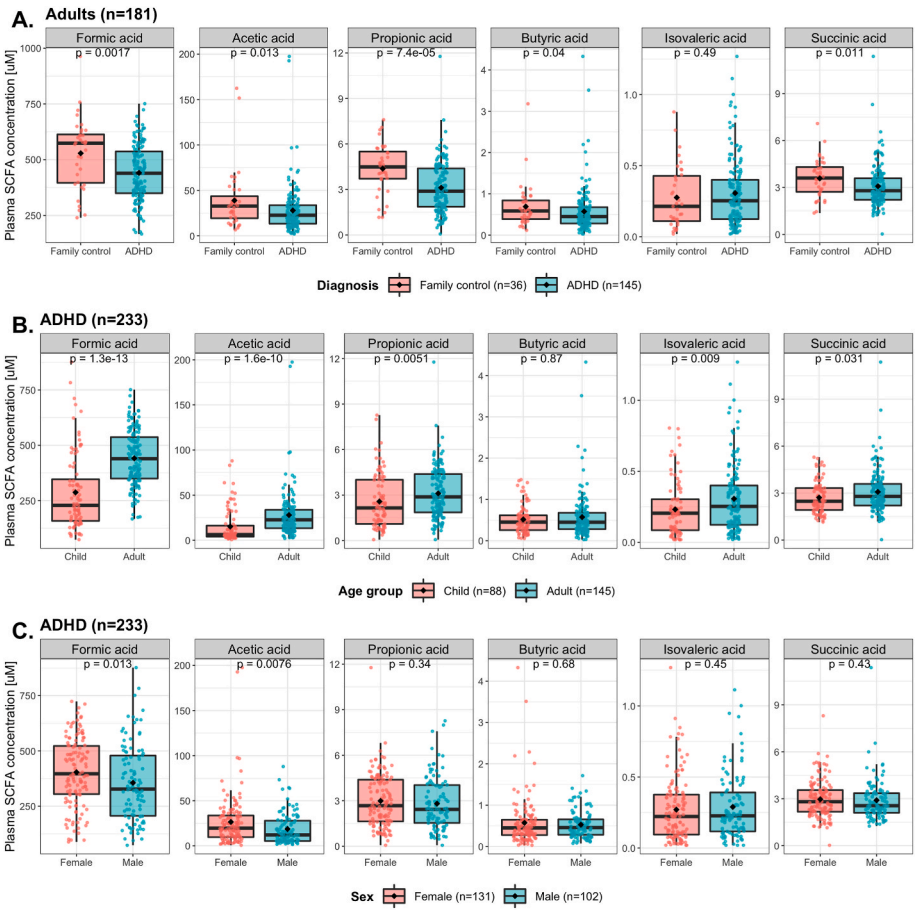

Fig. 1. Plasma levels of SCFAs in (A) adult patients with ADHD and adult healthy family controls, (B) children with ADHD and adults with ADHD, (C) female ADHD and male ADHD. Y-axes represent analyte levels with differences tested using nonparametric Mann–Whitney *U* test; Each dot represents a participant.

3.2. Determinates of SCFA concentrations in plasma from patients with ADHD

The aforementioned factors that were associated with levels of at least one of the SCFAs in the whole sample were included in a regression model for prediction of levels of individual SCFAs. To select the factors that fitted the final regression model on each SCFA, a backward elimination method was performed. As displayed in Table 2, the variances in SCFA level explained by the models were 30% for formic acid, 19% for acetic acid, 5.9% for propionic acid, 3.8% for butyric acid, 8.0% for isovaleric acid and 5.4% for succinic acid. The number of antibiotic doses taken during the last 2 years contributed to the model of all SCFAs, and more doses associated negatively ($p < 0.017$) with levels of formic acid, propionic acid and succinic acid explaining 2–4% of the variances in SCFA levels. Being a child contributed to lower formic acid, propionic acid and isovaleric acid levels explaining 27%, 4.2% and 5.6% of the variances, respectively. The full model did not reveal any additional association (Table S2). Thereafter, we studied if there was a difference in SCFA concentration between adult ADHD patients and adult healthy family members when controlling for number of antibiotic doses taken the last 2 years. First, we detected no difference in number of antibiotic doses between patients and healthy members (**Sup** Fig. S6A). Similar to ADHD patients, the number of antibiotic doses was negatively correlated with concentrations of acetic, propionic, butyric and succinic acid in family controls (**Sup** Fig. S6B). Finally, the concentrations of formic acid and propionic acid were lower in adult ADHD patients than in adult family controls also after adjusting for number of antibiotic drug doses (Table S3). Finally, we confirmed that the acetic and propionic acid levels in children on stimulant medication were lower also after adjustment for number of antibiotic doses the last 2 years ($p_{\text{acetic acid}} = 0.011$ and $p_{\text{propionic acid}} = 0.0090$, respectively).

4. Discussion

The gut microbiome has been suggested to be different in ADHD compared to healthy controls, a difference that could be influenced by host genetics as well as environmental exposures such as, but not limited to, delivery mode, age, diet, pharmaceuticals and stress (Cresci and Bawden, 2015; Hoyles et al., 2018; Nichols and Davenport, 2021). There are indications that these microbiome differences influence behavior in ADHD (Tengeler et al., 2020). As SCFAs are important mediators of the microbiota-gut-brain axis, putatively through the blood circulation, and SCFA levels in ADHD patients have not yet been reported, we here report plasma levels of six SCFAs (including the intermediary metabolite succinic acid) in children and adults with ADHD, and we estimate effect sizes on the SCFA levels of environmental factors influencing the gut microbiome. Indeed, we show that our cohort of adult ADHD patients had lower plasma levels of four of the five non-branched SCFAs, formic, acetic, propionic and succinic acid, compared to family controls, while the difference for butyrate ($p = 0.040$) did not reach statistical significance after correction of multiple testing. However, the SCFA concentrations were not associated with severity of core ADHD symptoms, autism symptoms or emotion dysregulation in our cohort (**Sup** Fig. S7 and Table S4). Non-branched SCFAs are derived mainly from dietary fibres, while the branched isovaleric acid is derived mainly from branched amino acids. Of the fibre-derived SCFAs, mainly propionate and butyrate have been reported to influence behavior of animal models. For example, intracerebroventricular injection with a high

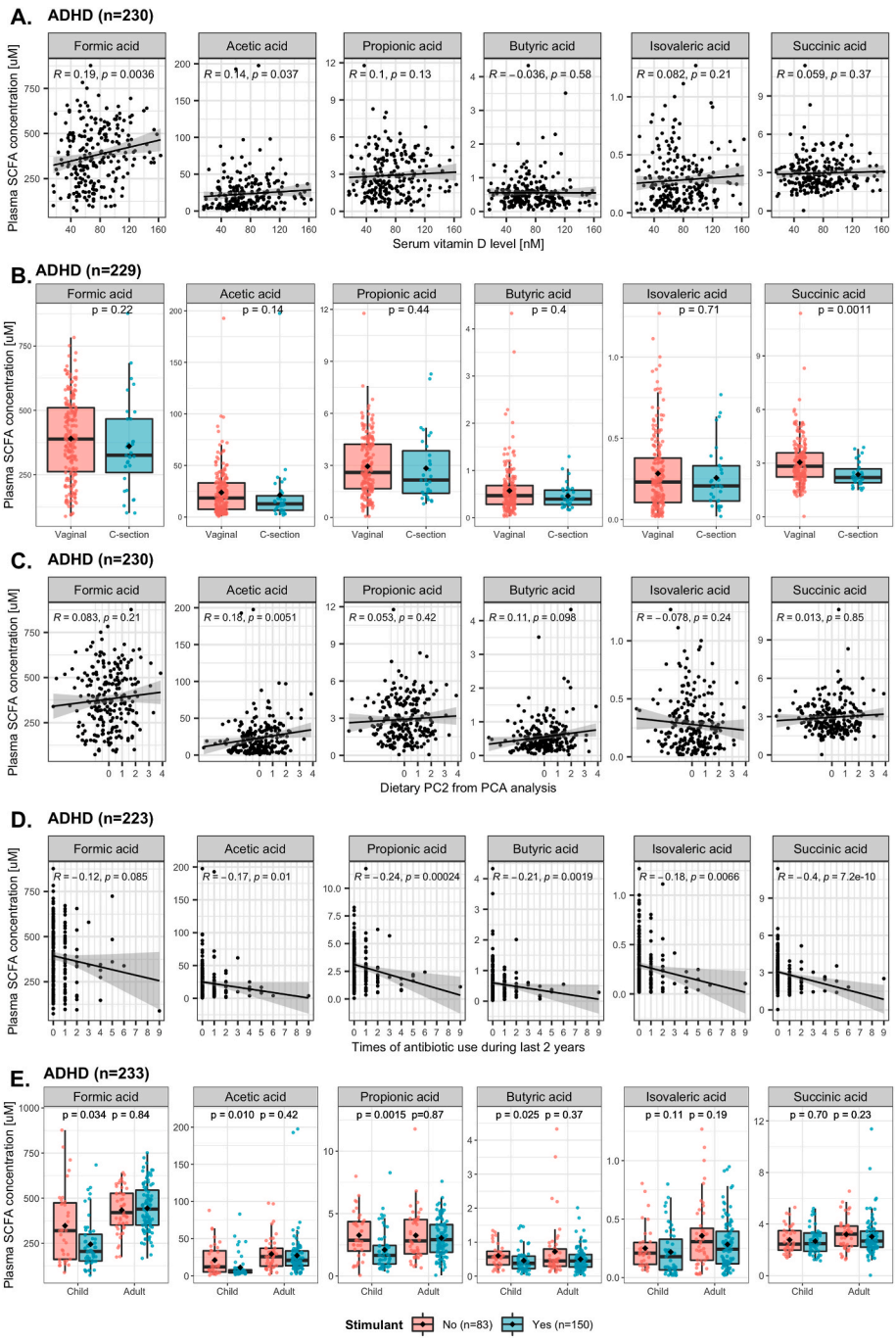

Fig. 2. Plasma levels of SCFAs in patients with ADHD in relation to (A) serum vitamin D levels, (B) delivery mode, (C) dietary PC2 and (D) antibiotic dose; (E) psychostimulant medication taken currently or 1–3 months ago. (A), (B) and (E): group differences were tested using nonparametric Mann–Whitney *U* test; (C) and (D): relationships were tested by Spearman's rank correlation showing the correlation coefficients and the corresponding p-values; the shaded area around the correlation line represents the 95% confidence interval; Y-axes represent analyte levels. Each dot in the plot represents a participant; Both children and adults are included. Some participants were excluded from some analyses due to unclear or missing information on antibiotic drug use (n = 10), serum vitamin D levels (n = 3) and delivery mode (n = 4).

concentration of propionate at 0.26 M (1.04 μmol) induced ASD (autism spectrum disorder)-like behavior (MacFabe, 2015; Shultz et al., 2008). Also, oral intake of butyrate at 1 g (9.1 mmol)/day or intestinal inoculation bacterial strains producing butyrate, acetate or propionate in germ-free mice was shown to restore blood-brain-barrier impairments (Braniste et al., 2014). Further, a combination of SCFAs (125 μmol propionate + 200 μmol butyrate + 337.5 μmol acetate) was reported to restore the immaturity and malformation of microglia in germ-free mice (Braniste et al., 2014; Erny et al., 2015). Moreover, SCFAs (acetic, propionic and butyric acids at μM level) have effects on proliferation, differentiation and gliosis of human neural cells (Abdelli et al., 2019; Yang et al., 2020a). The levels of SCFAs in human plasma are at μM levels (Human Metabolome Database, http://www.hmdb.ca/), that is, much lower than the range of SCFA concentrations in most reported models. It is plausible that SCFA levels far above physiological levels have effects different to those from high physiological levels. Reports on SCFA levels in plasma of psychiatric patients are few. Fecal SCFA levels, however, (acetic, butyric, isobutyric, valeric, isovaleric and caproic acids) were higher-than-normal in ASD (Wang et al., 2012). Lower fecal concentrations of propionate and butyrate have been reported in individuals with anorexia nervosa (Prochazkova et al., 2021). Notably, in multiple sclerosis, propionic acid concentrations are low in plasma, and increasing these levels reverses the regulatory T-cell/Th17 imbalance and improves disease course (Duscha et al., 2020). SCFA effects are further exemplified in **Supplemental Discussion**.

We identified in univariate models several factors putatively influencing the SCFA levels. Models were fitted in an attempt to identify non-confounded SCFA-influencing factors. Number of antibiotic doses taken

Table 2
Fitted regression model to predict plasma levels of SCFAs in ADHD patients.

	Estimate (η^2)					
	Formic acid	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	Succinic acid
Age group[1]	−0.53 (0.27)**	−0.86 (0.13)	−0.31 (0.042)*	–	−0.50 (0.056)*	–
Sex[1]	–	–	–	–	0.31 (0.023)	–
Vitamin D level[1]	–	0.21 (0.0090)	–	–	–	0.11 (0.010)
Delivery mode[1]	–	–	–	–	–	0.20 (0.020)
Dietary PC2	–	0.077 (0.0096)	–	0.082 (0.020)	- 0.096 (0.017)	0.11 (0.010)
Antibiotic dose[1]	−0.076 (0.031)**	−0.13 (0.023)	−0.12 (0.032)*	−0.10 (0.024)	−0.11 (0.020)	−0.086 (0.041)*
	adjusted R^2 (p)					
Fitted model[2]	0.30 (2.2e-16)**	0.19 (6.9e-10)**	0.038 (0.0064)**	0.059 (0.00056)**	0.080 (0.00024)**	0.054 (0.0021)**

Full model: ln[SCFA] = Age group + Sex + Vitamin D + Delivery mode + PC2 from dietary PCA + Antibiotic drug dose; [1.] Statistics for single variable; [2.] Statistics for fitted model; *p < 0.017, **p < 0.001.

the last 2 years associated negatively with levels of all SCFAs studied, and explained 2–4% of the variances in formic, propionic and succinic acid levels when adjusting for the other influencing factors studied in ADHD (Table 2, Fig. 2A). Antibiotic drugs can disrupt the normal composition and function of gut microbiota, such as SCFA production with long-lasting effects after stopping antibiotic intake (Holota et al., 2019; Willing et al., 2011). The succinic acid plasma concentration association with antibiotic drug use indicates that the microbiota is its significant source although succinic acid is produced also by the citric acid cycle in the host mitochondria (Martínez-Reyes and Chandel, 2020). Next, age group explained 27% of the variance in formic acid levels, and 4–5% of the variances of propionic acid and isovaleric acid, respectively, where SCFA levels were higher in adults than in children (Table 2, Fig. 1B). This may possibly be explained by the relationship between the gut microbiome composition and age although the diversity and abundance of gut bacteria resembles that of adult already at about 3 years of age and remains stable until old age (Cresci and Bawden, 2015). No other studied factor had a statistically significantly effect on SCFA concentrations among patients with ADHD when including all factors into the regression model (Table 2). In an attempt to explore if an ADHD diagnosis in adults influences the SCFA concentrations we compared the levels between patients and healthy family members among adults adjusting for number of antibiotic doses the last 2 years. Having a diagnosis of ADHD did influence the levels of formic and propionic acid when adjusting for number of antibiotic doses (Table S3). This was not due to current or recent medication. Notably however, the levels of acetic and propionic acids were lower in children on stimulant medication, also after adjusting for number of antibiotic doses taken the last 2 years (Fig. 2E). One study of fecal microbiome in adolescents and young adults with ADHD, 19 with and 22 without medication, found four genera to be decreased in the medicated group.(Szopinska-Tokov et al., 2020).

In the aforementioned univariate analyses, we found associations for some of the other studied factors with SCFA levels among the persons with ADHD. These factors have previously been reported to have a different distribution in ADHD patients compared to healthy controls (Del-Ponte et al., 2019; Kotsi et al., 2019; Shareghfarid et al., 2020; Zhang et al., 2019). First, diet can contain SCFAs and substrates for bacterial fermentation to SCFAs. The PC2, in which fruits, grain & whole grain cereals, vegetables and dairy had the largest loadings (0.17–0.56), associated with higher levels of acetic acid in univariate analysis (Fig. 2 and Sup Fig. S1). Thus, the PC with largest loadings of dietary fibres correlated with plasma concentration of one of the fibre-fermentation products, acetic acid (Martin-Gallausiaux et al., 2021). While we could not detect any diet difference between adult ADHD patients and healthy controls in our cohort, the children with ADHD had lower PC1 and PC2 than the adult patients. PC1 had, similar to PC2, high positive loadings of fruits, grain & whole grain cereals, vegetables, but fish and meat instead of diary, and negative loadings of sweets. Accordingly, recent meta-analyses reported a lower consumption of fruits and vegetables and higher consumption of refined sugar and saturated fat in children and adolescents with ADHD compared to controls (Del-Ponte et al., 2019; Shareghfarid et al., 2020), while for adult ADHD patients the findings are less clear (Holton et al., 2019; Li et al., 2020). Next, there was a positive association between formic acid and vitamin D levels in correlation analysis, but in the final fitted model the effect of vitamin D was not significant (Table 2, Fig. 2A). Age group might have masked a vitamin D-SCFAs correlation, as adults had higher levels of vitamin D than children (**Sup** Fig. S8). Both vitamin D and vitamin D receptor can regulate the homeostasis in the brain, the intestinal microbiome and the gut epithelial barrier (Ogbu et al., 2020; Yamamoto and Jørgensen, 2019). Delivery mode influences the establishment of the microbiome system of an infant (Cresci and Bawden, 2015). However, how long these influences last is unknown. In our univariate analysis, those with ADHD born vaginally had higher succinic acid levels than those born via C-section (Fig. 2B). The fact that this difference was driven by the children is reasonable as their births were more recent than the births of the adults (**Sup** Fig. 3A). Previous studies have reported associations between BMI and SCFAs (Kase et al., 2021), but in our study we did not find any such association. This is probably because most of our participants had a BMI in normal range (< 30 kg/m^2) (**Sup** Fig. S5D).

There were some limitations in this study. First, all the family controls were adults (n = 36), and there were only 18 patient-family control pairs among adults (**Sup** Fig. S2C). The pediatric sample was too small for a full regression model analysis. Second, the information for antibiotic use did not include ATC code, but the majority of them would probably be penicillin (ATC group J01C) (Lavebratt et al., 2019). Third, the food-frequency data was retrospective and did not cover all food items nor all food intakes. Fourth, BMI was available only from the adults. Fifth, we did not have information on host genetics and some environmental variables that have been showed to be associated with SCFA levels, such as smoking and blood pressure (Verhaar et al., 2020; Zeller et al., 2019).

5. Conclusion

Adult ADHD patients had lower plasma levels of formic acid, acetic acid, propionic acid and succinic acid than adult healthy family members. Using adjusted models we show that child patients had lower concentrations of formic, propionic and isovaleric acid than adult patients, and those who had more antibiotic medications during the last 2 years had lower concentrations of formic, propionic and succinic acid. After adjusting for antibiotic medication, we found that children currently on stimulant medication had lower acetic and propionic acid levels, and adults with ADHD had lower formic and propionic acid concentrations than adult healthy family members. In all, our findings show lower-than-normal plasma levels of SCFA in ADHD explained in-part by antibiotic medication, age and stimulant use. Whether or not this is of clinical significance is yet to be explored.

Author contribution

CL and LY conceptualized and designed the study. ES, MG and CL collected the clinical data and samples. LY, MS, TG and RL were involved in the measurements of the SCFAs. MS performed the analysis of diet data. LY undertook the statistical analysis and wrote the manuscript. LY and CL prepared the final version of the manuscript. All authors revised the manuscript and approved the final manuscript.

Funding source

This study was supported by the Swedish Research Council, Sweden (2014–10171 to CL), the Swedish Brain Foundation, Sweden (FO2020-0305 and FO2021-0412 to CL), Ekhaga Foundation, Sweden (2016–47), the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet, Sweden (SLL20190589 to CL).

Declaration of competing interest

The authors have no competing interests in relation to the work described.

Acknowledgement

We thank all the study participants, and research nurses Malena Kjellén from Karolinska Institutet and Åsa Söderström from PRIMA Child and Adult Psychiatry, and technician Rikard Fristedt from Chalmers University of Technology for SCFA concentration measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2022.09.042.

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