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Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders

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ABSTRACT

Evidence supporting that gut problems are linked to ASD symptoms has been accumulating both in humans and animal models of ASD. Gut microbes and their metabolites may be linked not only to GI problems but also to ASD behavior symptoms. Despite this high interest, most previous studies have looked mainly at microbial structure, and studies on fecal metabolites are rare in the context of ASD. Thus, we aimed to detect fecal metabolites that may be present at significantly different concentrations between 21 children with ASD and 23 neurotypical children and to investigate its possible link to human gut microbiome. Using ^{1}H-NMR spectroscopy and 16S rRNA gene amplicon sequencing, we examined metabolite profiles and microbial compositions in fecal samples, respectively. Of the 59 metabolites detected, isopropanol concentrations were significantly higher in feces of children with ASD after multiple testing corrections. We also observed similar trends of fecal metabolites to previous studies; children with ASD have higher fecal *p*-cresol and possibly lower GABA concentrations. In addition, Fisher Discriminant Analysis (FDA) with leave-out-validation suggested that a group of metabolites-caprate, nicotinate, glutamine, thymine, and aspartate-may potentially function as a modest biomarker to separate ASD participants from the neurotypical group (78% sensitivity and 81% specificity). Consistent with our previous Arizona cohort study, we also confirmed lower gut microbial diversity and reduced relative abundances of phylotypes most closely related to *Prevotella copri* in children with ASD. After multiple testing corrections, we also learned that relative abundances of *Feacalibacterium prausnitzii* and *Haemophilus parainfluenzae* were lower in feces of children with ASD. Despite a relatively short list of fecal metabolites, the data in this study support that children with ASD have altered metabolite profiles in feces when compared with neurotypical children and warrant further investigation of metabolites in larger cohorts.

1. Introduction

The prevalence of Autism Spectrum Disorders (ASD) continue to increase in the world [1,2]. Genetic, environmental and biological factors play a critical role in neurodevelopment during a mother's pregnancy and immediately after birth [3,4]. The comorbidity of GI

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symptoms (primarily chronic constipation and/or diarrhea) in ASD is generally estimated to be 30–50%, and is poorly understood [5,6]. The significantly increased presence of GI symptoms in ASD has motivated researchers to explore gut microbial composition in children with ASD and hypothesize about their potential role in contributing to/reflecting ASD symptoms [7–10].

Researchers have focused on whether children with ASD possess a lack of beneficial or an increase of harmful microbes in their gut. Potentially harmful *Clostridium* species were observed abundant in feces of children with ASD [11–13]. One type of beneficial bacteria, i.e., *Bifidobacterium*, was reduced in children with ASD [14–16], while another probiotic, i.e., *Lactobacillus*, was reported to be present in higher concentrations in children with ASD [9,16]. Using next-generation sequencing technology, microbes that might otherwise be ignored have been detected and proposed as either potentially beneficial bacteria (e.g., *Prevotella*) [7] or harmful bacteria (e.g., *Sutterella*) [17] in the context of autism. Observations on individual bacterial taxa, however, have not always been congruent between studies. For example, *Desulfovibrio* and *Akkermansia* levels in children with ASD were found to be either higher [10,14] or lower [7,15], although multiple testing corrections was not always performed [10]. In some cohorts, no difference in gut microbiota was reported between children with ASD and their neurotypical siblings [18,19], and some studies have found differences between individuals with ASD and their siblings [14], but neurotypical siblings may be different from the general neurotypical population. As previously reviewed [20], these discrepancies may be attributed to the broad spectrum of ASD manifestations, varying molecular technologies used to investigate subject samples, geographical differences between participants (which may result in genetic and/or dietary differences), potential sub-types of gut microbiota within ASD groups, small sample size and inadequate statistical control for testing multiple-hypotheses. Considering the redundant functions of microbes [21], research must expand beyond simply cataloging microbial composition and instead also investigate microbial functions and interrelated pathways, since important microbial functions may be masked when gut microbes are considered individually.

Studies conducted in humans [5,6,16] and animal models of ASD [22–26] suggest that gut microbes and their metabolites may be linked not only to GI problems but also to ASD behavior symptoms. Hsiao et al. [22] demonstrated that 4-ethylphenylsulfate (4EPS) and indolepyruvate concentrations in serum were strikingly increased in the maternal immune activation (MIA) mouse model that displays ASD symptoms and induced anxiety-like behaviors in offspring mice. *Bacteroides fragilis* modulated 4EPS concentrations most likely by restoring tight-junction integrity [22]. Likewise, stress-induced corticosterone levels were reduced when stressed mice were treated with *Lactobacillus rhamnosus* [25]. The treatment with beneficial bacteria, *Lactobacillus reuteri*, ameliorated deficient social behaviors in maternal high-fat diet offspring mice, and oxytocin levels were restored [24]. Oxytocin is a crucial hormone in social behavior and cognition [27], but its therapeutic effect on ASD needs more human clinical trials to draw definite conclusions [28,29].

Despite these compelling observations, studies that examine human gut microbial metabolites are rare in the context of ASD. Functional level analyses such as metagenomics, metatranscriptomics, and metabolomics should follow studies of microbial composition: metabolomics has the advantage that it can provide information about the final products of microbial functions. Metabolomics with fecal samples can provide clues on gut microbial metabolism. However, most metabolomics studies focus on urine and blood metabolites [30–35]. Only a few studies have investigated fecal metabolites and attempted to correlate them with gut microbial structure in children with ASD. Wang et al. [36] observed elevated short-chain fatty acids (SCFA) and ammonia concentrations in feces of children with ASD, although De Angelis et al. [14] and Adams et al. [16] reported reduced total SCFA in children with ASD. De Angelis et al. [14] also observed altered levels of neurotransmitters-glutamate and GABA in fecal samples. Glutamate was highest in children with autism and GABA was lowest in children with Pervasive developmental disorder not otherwise specified (PDD-NOS). In addition, phenol substances including *p*-cresol were higher in feces of children with autism and PDD-NOS [14], but phenol and *p*-cresol levels were comparable between children with ASD and controls in the other cohort [36].

In this study, we received fecal samples from children with ASD and neurotypical controls, and obtained quantitative levels of 59 fecal metabolites and gut microbial profiles using ^{1}H-NMR spectroscopy and pyrosequencing, respectively. We detected some fecal metabolites that may be present at significantly different concentrations between children with ASD and neurotypical children. We also performed correlation tests between fecal metabolite and individual bacterial phylotypes, and postulated their potential implications in the detection, etiology, and treatment of GI and ASD symptoms in children with ASD.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Review Board (IRB) at Arizona State University (ASU IRB Protocol #: 1206007979). We advertised the study by email to families with family members with ASD in Arizona, USA, and mailed the consent form to people who showed an interest in participating in the study. Once the signed consent forms were returned, we screened them for eligibility criteria and sent questionnaires and sample collection kits to participants.

2.2. Subject recruitment and sample collection

21 neurotypical children and 23 children with ASD were recruited for the study. Among them, 3 neurotypical children and 6 children with ASD were re-invited from the cohort of a previous study [7], and fresh samples and new questionnaires were collected. The participants were between the ages 4–17 years old, and the severity of GI symptoms were assessed using the 6-item GI severity index (6-GSI). Children with ASD were additionally assessed with Autism Treatment Evaluation Checklist (ATEC) and Pervasive Developmental Disorder Behavior Inventory (PDD-BI). Neurotypical children did not have a first degree relative with a diagnosis of ASD. ASD and neurotypical children were enrolled regardless of any GI symptoms. We also obtained the participants' medical histories, gastrointestinal problems, diet and use of medications including nutritional supplements. The participants did not use antibiotics and antifungal medications in the month prior to sample collection. The parents of the participants collected fecal samples as directed in the instruction provided and froze them immediately until shipment. The participants shipped frozen fecal samples overnight to Arizona State University with cold packs. Once received, fecal samples were stored in −80 °C freezer.

2.3. Metabolite extraction and ^{1}H-NMR metabolomics analysis

We aliquoted 0.5 g of semi-frozen fecal sample, and added 10 mL of sterile 18.2-ohm water. We vortexed them at highest speed for 3 min until the sample was completely homogenized. We centrifuged at 13,000 rpm at 4 °C for 15 min to settle the solid particles,

then filtered the supernatant through 0.2 μm pvdf membrane filter disk (PALL corporation, Ann Arbor, MI, USA). We mixed 270 μL of the fecal extracts with 30 μL of ^{1}H-NMR reference mix, and transferred the mixtures into 3 mm NMR tubes. ^{1}H-NMR reference mix consisted of 0.022436 g of DSS-d6 and 0.04 g sodium azide in 10 mL 100% deutreum oxide. We processed autism and control samples in a random order under the same condition in order to avoid any lab- and instrumental-related bias.

The resulting mixture was sent to Pacific Northwest National Laboratory (PNNL) for ^{1}H-NMR analysis. At PNNL, data were acquired on a Varian Direct Drive (VNMRS) 600 MHz spectrometer (Agilent Technologies). The spectrometer system was outfitted with a Varian triple resonance salt-tolerant cold probe with a cold carbon preamplifier. A Varian standard one dimensional proton nuclear Overhauser effect spectroscopy (noesy) with presaturation was collected on each sample, using a nonselective 90° excitation pulse, a 100 millisecond mixing time, acquisition time of 4 s, a presaturation delay of 1.5 s, spectral width of 12 ppm, and temperature control set to 25 °C. Collected spectra were analyzed using Chenomx 8.0 software, with quantifications based on spectral intensities relative to 0.5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 (Sigma-Aldrich), which was added as a spike to each sample. With respect to the ^{1}H-NMR metabolite data, we calculated the average of the duplicates for each compound and normalized individual metabolite levels to per gram dry-weight feces.

2.4. DNA extraction and 16S rRNA sequencing analysis

We extracted genomic DNA using a PowerSoil DNA extraction kit with a modification based on the manufacture protocol. In order to enhance a cell lyses, we additionally performed a 65 °C incubation for 10 min after adding C1 solution. We sent genomic DNA samples to the Research and Testing laboratory (Lubbock, TX, USA), where next-generation sequencing was performed with the Genome Sequencer FLX-Titanium System (Roche, Indianapolis, IN). We selected bacterial primers 104F (5′-GGCGVACGGGTGAGTAA-3′) and 530R (5′-CCGCNGCNGCTGGCAC-3′) to target V2-V3 regions of 16S rRNA genes and the amplicons were sequenced as described in Wolcott et al. [37]. We performed sequencing analysis using the Quantitative Insights Into Microbial Ecology (QIIME) software package [38]. Sequences were demultiplexed and qualified by QIIME's split_libraries_fastq.py. We then employed pick_open_reference_otus.py with the Greengenes 13_5 reference database and obtained an operational taxonomic units (OTU) table by clustering sequences at sequence similarity of 97% using UCLUST clustering algorithm [39]. Chimeric sequences were identified and removed from the OTU table by using ChimeraSlayer [40]. OTU representative sequences were taxonomically assigned using the RDP Classifier [41]. We calculated within-sample (alpha) and inter-sample (beta) diversity indices at the rarefaction depth of 8,273 that is the minimal number of sequence reads throughout the samples. To estimate microbial diversity within samples (alpha diversity), we employed a non-phylogenetic metric *Observed OTUs* that counts operating taxonomic units (OTUs) given a sample depth and Faith's phylogenetic diversity (PD) index that estimates the tree-branch lengths on the phylogenetic cladogram to embrace phylogenetic relationship [42] using QIIME software package [38]. We also measured the phylogenetic distance and similarities between samples by using unweighted (which equally weights taxa regardless of their relative abundance) as well as weighted (in which the more abundant species are weighted more heavily) UniFrac analyses [43].

In parallel, we also predict bacterial metabolic pathways and functional genes using the online Galaxy version of the PICRUSt software package [44]. PICRUSt was used to predict a shotgun metagenome of KEGG Orthologs (KOs) for the detected OTUs with GreenGenes reference sequences. Before metagenome prediction, the OTU table was first rarefied to 6011 reads per sample. Predicted genes were then grouped into pathways and these were tested for significant difference in abundance between autistic and neurotypical individuals using the Mann-Whitney *U* test. Targeted analysis of the production of specific metabolites was done using PICRUSt and the KEGG database [45]. We extracted the following information from KEGG files using custom python code: 1) molecules of interested were found in the Compound file, 2) the reactions that produce these molecules were found in Reaction file, and 3) Genes that catalyze these reactions were identified in the KO file. We then looked for these genes in our PICRUSt data to determine if they were statistically significantly differentially abundant between the two groups via the Mann-Whitney *U* test.

2.5. Statistical analyses

Statistical analyses were done by Python (SciPy and Statlib packages) and R programming (a function "p.adjust" in R stats package for multiple testing correction; the caTools package for obtaining the area under the curve (AUC) values). Since our sample size is small (n = 21 and 23 for neurotypical children and children with ASD, respectively), we employed the non-parametric Mann Whitney *U* test to assess the difference in metabolites and relative abundance of microbial taxa. Out of 59 fecal metabolites, five metabolites (2-hydroxyisobutyrate, asparagine, homovanillate, tyramine, and urea) were detected only in zero to two samples (see Dataset S1), and those unqualified metabolites were not included for downstream statistical analysis. We also employed the permutation test by randomly shuffling individual metabolite dataset 10,000 times by Python (the random module). With 10,000 shuffled dataset, we performed the non-parametric Mann-Whitney *U* test, and obtained *p* values for individual metabolites. For hypothesis-driven tests, we accepted one-tailed *p* values less than 0.05 as significant and considered *p* values between 0.05 and 0.10 as marginally significant. For exploratory tests, we adjusted *p* values after correction for multiple testing using the Benjamini & Hochberg method [46] and considered two-tailed *p* values less than 0.05 to be significant. For correlation tests, we conducted non-parametric Spearman's rank-order correlation test and controlled false discovery rates by using Benjamini & Hochberg method [46]. We standardized distributions of two different datasets by converting them to z-scores (z=(x-m)/s, m = average, s = standard deviation) and calculated the correlation between fecal metabolites and individual bacterial taxa. We accepted adjusted *p* values less than 0.05 as significant for the Spearman correlation test. For the network analysis, we used Cytoscape [47] to visualize the correlations we obtained by the Spearman correlation test.

Fisher Discriminant Analysis (FDA) was used to search for sets of variables that could distinguish between ASD and controls. The FDA was performed with custom scripts in MATLAB 2016b. FDA finds the linear projection that simultaneously maximizes the between-class scatter and minimizes the within-class scatter by maximizing the objective function

$$J = w^T S_B w \Big/ w^T S_W w$$

where w is the projection vector, S_B is the between-class scatter, and S_W is the within-class scatter. To guard against overfitting, leave-one-out cross-validation is employed, where one sample is left out and the remaining samples are used to generate a FDA model. Then, the classification performance on the left-out sample is recorded and the process is repeated for the remaining samples.

In this way, each sample is assessed on a FDA model that had not used that sample for training, generating a statistically independent assessment.

3. Results and discussion

3.1. Subject characteristics

21 neurotypical children and 23 children with ASD participated in the study (Table 1). All children were between 4 and 17 years old, with a mean age (±SD) of 8.4 (±3.4) years for neurotypical children and 10.1 (±4.1) years for children with ASD (Table 1). We assessed the severity of GI symptoms using the 6 item Gastrointestinal Severity Index (6-GSI) [16], and GI symptoms were significantly more severe for children with ASD compared to controls (two-tailed Mann-Whitney *U* test, $p < 0.005$) (Table 1). The 6-GSI scores were strongly and positively correlated with the ATEC, especially when using a subscale of Health/Physical/Behavior (Fig. 1AB, Spearman rank correlation coefficient $r = 0.45$ and 0.54; $p = 0.03$ and < 0.01 for ATEC Total and ATEC-Health/Physical/Behavior, respectively.), which is consistent with previous observations [16].

3.2. Isopropanol concentrations were significantly higher in feces of children with ASD

We used two approaches to analyze the metabolomics dataset—an exploratory and a hypothesis-driven investigation. Here we discuss the results of the exploratory investigation, and below we discuss the results of hypothesis-driven investigations. In order to discover any new metabolite that could be a signature for ASD phenotype, we calculated *p*-values for all metabolites and then employed multiple testing corrections on them. Among 59 metabolites detected, isopropanol (or propan-2-ol) was the only metabolite that was significantly different between the ASD and neurotypical groups after multiple testing corrections (Permutation-based non-parametric significance test, adjusted $p = 0.022$, Table 2). We confirmed that our observation on isopropanol is not of a laboratory contaminant. Isopropanol was not involved in any experimental procedure from the fecal extraction to ^{1}H-NMR measurement, and we did not observe a similar peak belonging to isopropanol in our blank negative samples. It is intriguing to see a significantly higher level of isopropanol concentrations in feces of children with ASD (82% higher in children with ASD based on average values), considering isopropanol is a neurotoxic organic solvent that contributes to central nervous system and respiratory depression [48]. To our knowledge, isopropanol has not been investigated in previous studies of children with ASD. Isopropanol is a common rubbing alcohol and widely used as a sanitizer, but its absorption through skin is limited [49]. However, isopropanol is rapidly absorbed through the gastrointestinal tract [50]. A possible relationship between isopropanol and ASD is unknown, but isopropanol poisoning irritates mucosal surfaces and causes GI problems such as abdominal pain [51]. Higher isopropanol levels in feces of children with ASD may be attributed to higher bacterial activity that converts acetone to isopropanol through isopropanol dehydrogenase [52]. *Lactobacillus brevis*, *Clostridium beijerinckii*, and *C. aurantibutyricum* are microbes that produce isopropanol from acetone [53–56], but we did not detect any of those microbial species either in feces of children with ASD or neurotypical children. There may be other bacteria which produce isopropanol in the human gut, and this needs further investigation.

Table 1
Summary of subject characteristics.

Subject characteristic[a]	Neurotypical	Autistic
Total # participants (# re-visit participants)	21 (3)	23 (6)
Male/Female	15/6	22/1
Age (years)	8.4 ± 3.4[b]	10.1 ± 4.1
ATEC	–	62.3 ± 29.1
PDD-BI	–	−60.6 ± 66.4
6-GSI	2.1 ± 2.2	4.5 ± 2.9

[a] Detailed information including GI severity, autistic severity indices, and diet survey are attached in the Dataset S1.
[b] All values are mean ± standard deviation.

3.3. p-cresol was consistently higher in feces of children with ASD

Although only isopropanol was at significantly different concentration in ASD vs. controls after multiple test correction, we continued investigating other metabolites that have been of interest in previous studies. These were hypothesis-driven investigations, hence no correction for multiple hypothesis testing was used. In previous studies, *p*-cresol levels were significantly higher in blood, urine, and feces of children with ASD [14,34]. Consistently, we also observed that *p*-cresol concentrations were significantly higher in feces of children with ASD compared to neurotypical children (26% higher in autistic samples; one-tailed Mann-Whitney *U* test, un-adjusted $p = 0.04$). *p*-cresol is either introduced through skin or inhalation [57] or microbially produced in the intestine from dietary tyrosine or toluene [58]. We, however, found no significant correlation between *p*-cresol and tyrosine in feces (Spearman correlation $r = -0.08$, $p = 0.62$). Dietary intervention studies [59] show that the amount of resistant starch in the diet is inversely associated with the amount of *p*-cresol in feces and urine. In addition to its potential roles in chronic kidney disease, cardiovascular disease, and colorectal cancer [60–62], *p*-cresol seems to negatively affect the homeostasis of colonic epithelial cells in children with ASD. When present in excess, *p*-cresol induces DNA damage *in vitro* and deleteriously affects colonic epithelial cell integrity [63]. In ASD mouse model experiments, Hsiao et al. [22] observed that 4-ethylphenylsufate (4EPS) was higher in the serum of the ASD mouse model, and was normalized after *B. fragilis* treatment. This treatment also changed the gut microbiota and improved gut permeability and ASD-like behaviors [22]. The authors underscored a structural similarity and biosynthesis pathway shared between 4EPS and *p*-cresol sulfate, a derivative form of *p*-cresol. Since *p*-cresol is converted to *p*-cresol sulfate by host sulfation, further studies on both *p*-cresol and *p*-cresol sulfate in matched human feces, blood, and urine samples are warranted.

3.4. Propionate and butyrate concentrations were comparable between feces of children with ASD and neurotypical children

We hypothesized that children with ASD would have higher propionate and lower butyrate concentrations in their feces, since intraventricular infusions of propionate induced ASD-related behaviors in animal studies [23,64] and butyrate has a beneficial role in the human intestine [65,66]. However, propionate and butyrate were not different between our control and ASD cohorts (Table 2). In fact, previous studies on the major SCFAs- propionate, butyrate, and acetate-in ASD have been mixed: fecal concentrations of SCFA were reported to be either lower [14,16] or higher [36] in children with autism. The other carbohydrate fermentation products of intestinal bacteria, for example of formate and lactate, were detected at relatively lower concentrations in children with ASD (Table 2), but their differences were not significant after multiple testing corrections.

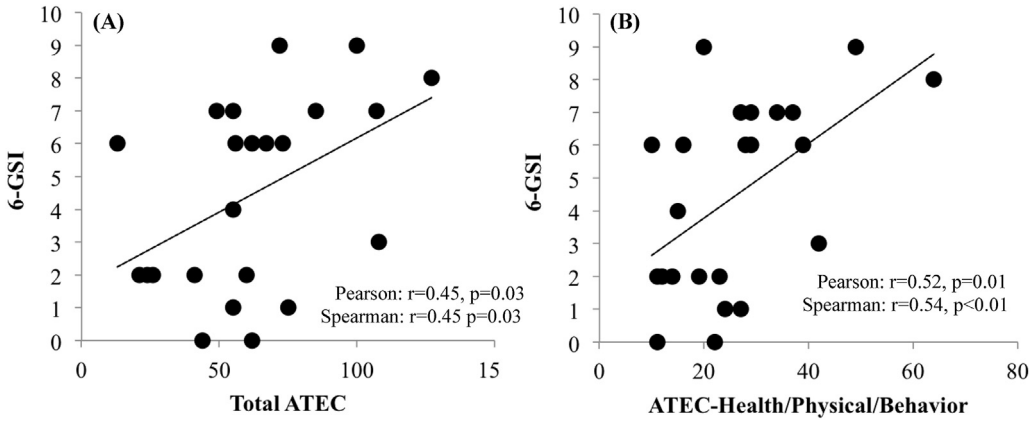

Fig. 1. Correlation between GI symptoms' severity and autism behaviors (A. Total ATEC, B. ATEC subscale Health/Physical/Behavior).

Table 2
Comparison of fecal metabolites and pH between neurotypical 5 and autistic samples.

Metabolites[a]	Mann-Whitney *U* *p* values[b]		Permutation *p* values[b]		AUC[c]	Median[d] (25%/75%)	
	un-adjusted	adjusted	un-adjusted	adjusted		Neurotypical (n = 21)	Autistic (n = 23)
Isopropanol	0.001	0.057	<0.001	0.022	0.788	0 (0/0.12)	0.15 (0.11/0.29)
Lactate	0.026	0.716	0.026	0.631	0.697	0.32 (0.21/0.46)	0.13 (0.07/0.29)
Formate	0.041	0.716	0.034	0.631	0.681	0.99 (0.73/1.17)	0.67 (0.56/0.80)
p-cresol	0.082	0.716	0.079	0.708	0.654	1.65 (1.27/2.08)	2.03 (1.61/2.81)
Caprate^{+}	0.091	0.716	0.086	0.708	0.650	0.72 (0.6/0.9)	0.9 (0.7/1.2)
GABA	0.154	0.716	0.148	0.708	0.622	0.11 (0.04/0.25)	0 (0/0.19)
Nicotinate	0.173	0.716	0.173	0.708	0.621	0.66 (0.42/1.32)	0.52 (0.33/0.86)
GABA/glutamate	0.194	0.716	0.168	0.708	0.616	0.01 (<0.01/0.01)	0 (0/0.01)
Glutamine^{+}	0.205	0.716	0.202	0.708	0.613	1.98 (1.47/2.63)	1.91 (1.16/2.23)
Thymine	0.250	0.776	0.237	0.738	0.602	0.49 (0.38/0.88)	0.39 (0.26/0.66)
Glutamate	0.301	0.868	0.296	0.738	0.592	17.78 (11.61/22.04)	13.68 (9.91/19.05)
Aspartate	0.385	0.946	0.376	0.915	0.578	3.47 (1.68/6.58)	4.33 (2.42/8.66)
Butyrate	0.452	0.974	0.445	0.960	0.567	37.15 (25.36/50.20)	32.55 (22.85/43.65)
Acetate	0.622	0.979	0.603	0.960	0.545	162.39 (123.12/222.97)	158.42 (115.93/187.96)
Propionate	0.638	0.979	0.628	0.960	0.542	39.20 (32.32/66.78)	38.83 (30.44/55.49)
Tyrosine	0.906	0.979	0.889	0.960	0.511	4.00 (2.92/6.16)	5.09 (2.54/6.46)
Fecal pH	0.912	0.979	0.902	0.960	0.510	6.84 (6.40/7.56)	6.72 (6.56/7.25)

[a] Detailed information with a whole list of metabolites are listed in the Dataset S1.
[b] P values are two-tailed un-adjusted and adjusted from Mann-Whitney *U* test and permutation test. For hypothesis-driven tests, we accepted two-tailed *p* values less than 0.05 as significant and considered *p* values between 0.05 and 0.10 as marginally significant.
[c] Area under curve (AUC) values of 0.5 indicate no predictive value, while an AUC of 1 indicates perfect ability to predict. The values between 0.5 and 1.0 are predictive values.
[d] Unit: μmole per g-dry stool $^{+}$Tentative assignment.

3.5. GABA concentrations trends were lower in feces of children with ASD

A possible link of gut microbes and brain function is of high interest and there is emerging evidence that a dysfunction in balancing two major neurotransmitters-gamma-Aminobutyrate (GABA) and glutamate-is linked to autism symptoms, particularly impairments in sensory processing (e.g. hyper/hyposensitivity to touch) [67]. De Angelis et al. [14] observed altered fecal concentrations of neurotransmitters-glutamate and GABA, where glutamate was highest in children with autism and GABA was lowest in children with PDD-NOS. We wanted to determine whether this observation was replicated in our cohort of children with ASD.

First, GABA, a major inhibitory neurotransmitter, was in lower concentration in the feces of children with ASD in our cohort, although the difference was only marginally significant (36% lower in autistic samples; one-tailed Mann-Whitney *U* test un-adjusted $p = 0.077$). However, concentrations of glutamate, a major excitatory neurotransmitter, were comparable between groups. The GABA to glutamate ratio is a signature of neuroinflammation [68] and a measure of the balance between inhibitory and excitatory neurotransmiters, and we observed this ratio to be marginally lower in feces of children with ASD (one-tailed Mann-Whitney *U* test un-adjusted $p = 0.097$). Wu et al. [69] reported that microbial symbiosis improved GABA production in *in vitro* experiments, where *Streptococcus thermophilus* enhanced glutamic acid decarboxylase (GAD) gene expression in *Lactobacillus brevis*, a GABA producer [69]. We observed that relative abundance of *Streptococcus* were significantly higher in feces of neurotypical children (one-tailed Mann-Whitney *U* test, un-adjusted $p = 0.03$), although its correlation with GABA concentrations was weak (Spearman correlation $r = 0.16$, $p = 0.31$). Metagenomics and metatranscriptomics analyses focusing on the GABA-glutamate system are warranted to provide mechanistic information regarding how gut microbiota can contribute to modulating GABA and glutamate levels in children with ASD.

We performed correlation tests between fecal metabolites highlighted above and clinical assessment scores, but did not find any significant correlation between fecal concentrations of metabolites and total scores of 6-GSI, ATEC, and PDDBI assessments after

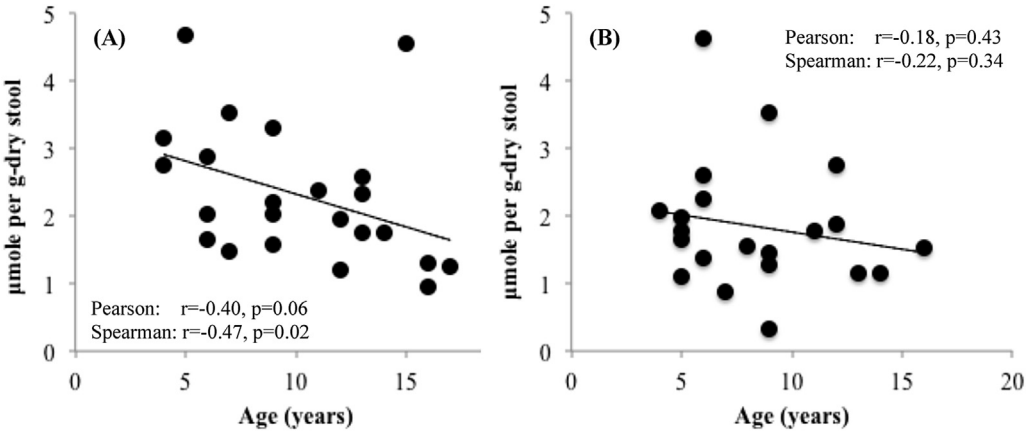

Fig. 2. Correlation between age and concentrations of *p*-cresol in feces of (A) children with ASD and (B) neurotypical children.

multiple testing correction (Data not shown). Intriguingly, however, *p*-cresol concentrations were significantly and negatively correlated with age within the ASD group (Fig. 2A, Spearman correlation, $r = -0.47$, $p = 0.02$), whereas no significant correlation was observed within the neurotypical children group (Fig. 2B, Spearman correlation, $r = -0.22$, $p = 0.34$); this suggests that *p*-cresol concentrations might be even higher in ASD vs. controls at younger ages. In a previous study, total urinary *p*-cresol was significantly higher especially among younger children with ASD and was proposed as a potential biomarker [34]. Higher *p*-cresol in younger age children may reflect earlier exposure to *p*-cresol or its precursors and a premature GI environment leaning toward more bacterial *p*-cresol production. We investigated if other fecal microbial metabolites were also correlated with age. Trimethylamine, malonate, methylamine, choline, and 3-phenylpropionate were weakly correlated with age within the ASD group, but not statistically significant after correction for multiple hypothesis testing (Table S1).

3.6. Based on fecal metabolites, Fisher Discriminant Analysis suggests separation between ASD and neurotypical group

Differences in individual fecal metabolites suggested that metabolite measurements might be useful as indicators/biomarker for separating the participants into autism and neurotypical groups. Therefore, we used Fisher Discriminant Analysis (FDA) with variable selection to build this classifier. We evaluated metabolite combinations from the top 25 most differently abundant metabolites. Combinations with the same number of metabolites selected were evaluated based on the fitted objective function value, which is a measure of how well the classifier separates the two groups. The top 100 combinations for each number of metabolites were evaluated using cross-validated sensitivity and specificity. The final FDA model is presented in Fig. 3 and Fig. S1 (the values of Probability Density Functions (PDFs) are in Fig. 3A) and includes caprate, nicotinate, glutamine, thymine, and aspartate (Fig. 3B). It is important to note that other combinations provided similar results (Table S2), but only the best combination (Fig. S2) is discussed further for clarity. Using a threshold of >20% membership in the ASD class, cross-validation results indicate that based on this analysis only 5 out of 23 children with ASD would not be classified as belonging to their true groups (78% sensitivity) and 4 out of 21 neurotypical children would be misclassified (81% specificity). Furthermore, using a threshold of >30% membership in the ASD class, cross-validation indicates 70% sensitivity and 95% specificity.

Fig. 3B shows how the objective function value *J* (described more in Methods) changes when each variable is sequentially left

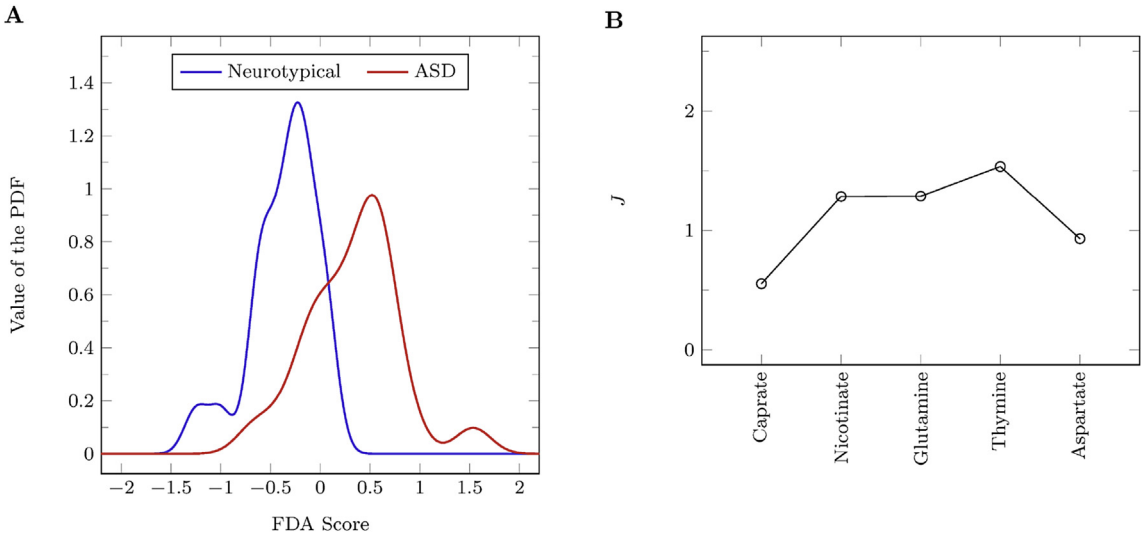

Fig. 3. Fisher Discriminant Analysis (FDA) model using caprate, nicotinate, glutamine, thymine, and aspartate: (A) Probability Density Functions (PDFs) of neurotypical and ASD participants and (B) objective function value *J* when each variable is left out.

Table 3
Alpha diversity (*Observed OTUs* and PD) was significantly lower in feces of children with ASD compared with neurotypical children.

	Two-tailed Mann-Whitney *U* test *p* value[a]	Median (25%/75%)	
		Neurotypical (n = 21)[b]	ASD (n = 23)
Observed OTUs	<0.001	990 (911/1,098)	763 (586/918)
PD index	<0.001	61 (55/67)	48 (39/58)

[a] *p* values are from two-tailed Mann-Whitney *U* test in order to test the hypothesis.
[b] In Table S3, we also compared alpha diversity between 18 neurotypical 17 autistic samples after 9 re-invited samples (3 neurotypical and 6 autistic) were excluded. The differences on alpha diversity were still significant (Table S3, -23% Observed OTUs and −19% PD; one-tailed Mann- Whitney *U* test $p = 0.001$ and 0.004 for *Observed OTUs* and PD, respectively).

out, and reflects each variable's contribution to the fitted separation. Among the variables for the developed FDA model, concentrations of caprate and aspartate were higher in feces of children with ASD, whereas nicotinate, glutamine, and thymine were lower in ASD groups (Table 2). Among the 5 metabolites, caprate was the most important contributor to the model (Fig. 3B), and concentrations of caprate were marginally higher in feces of children with ASD (29% higher in autistic samples; two-tailed Mann-Whitney *U* test, un-adjusted $p = 0.091$). Caprate (deconic acids) is a major component of a ketogenic diet, which is known to be effective at controlling epilepsy and seizure [70]. Its therapeutic role may be attributed to its inhibitory activity to excitatory neurotransmission [71].

Among the variables for the developed FDA model, concentrations of aspartate, nicotinate, glutamine, and thymine were not significantly different between groups based on Mann-Whitney *U* test (Table 2). However, the multivariable methods employed here may allow us to uncover relationships in the metabolite data, which were not captured by univariate hypothesis testing (e.g. Student's t-test or Mann-Whitney *U* test). The second most contributing variable, aspartate, is an excitatory neurotransmitter and acts on N-methyl-D-aspartate (NMDA) receptors [72]. Previously, De Angelis et al. [14] reported higher levels of aspartate in feces of children with ASD. We also observed relatively higher concentration in our ASD cohort, which may reflect its potential contribution to ASD symptoms associated with NMDA receptor dysfunction [73]. Glutamine is another neurotransmitter-related metabolite, as it is a precursor for two major CNS neurotransmitters (glutamate and GABA) [74]. Regarding nicotinate, Yap et al. [33] observed increased levels of nicotinate derivatives in urine samples from individuals with autism, and suggested alterations in tryptophan-nicotinate metabolism. Tryptophan is a precursor for both nicotinate and serotonin, and increased levels of nicotinate indicate a reduced conversion of tryptophan to serotonin that is an important neurotransmitter known as beneficial for human health multiple routes [75]. Adams et al. [30] found that nicotinate (niacin) concentration in urine of children with ASD were normal, but concentrations of the active forms-reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH)- were significantly lower. Although potential biological roles require mechanistic verification, our FDA results indicate that multivariate interactions may be important for interpreting this type of data and that the selected metabolites may potentially function as biomarkers to classify ASD cases. The "leave-one-out" validation helps to ensure that the FDA results are robust, and will apply to other individuals that did not participate in this

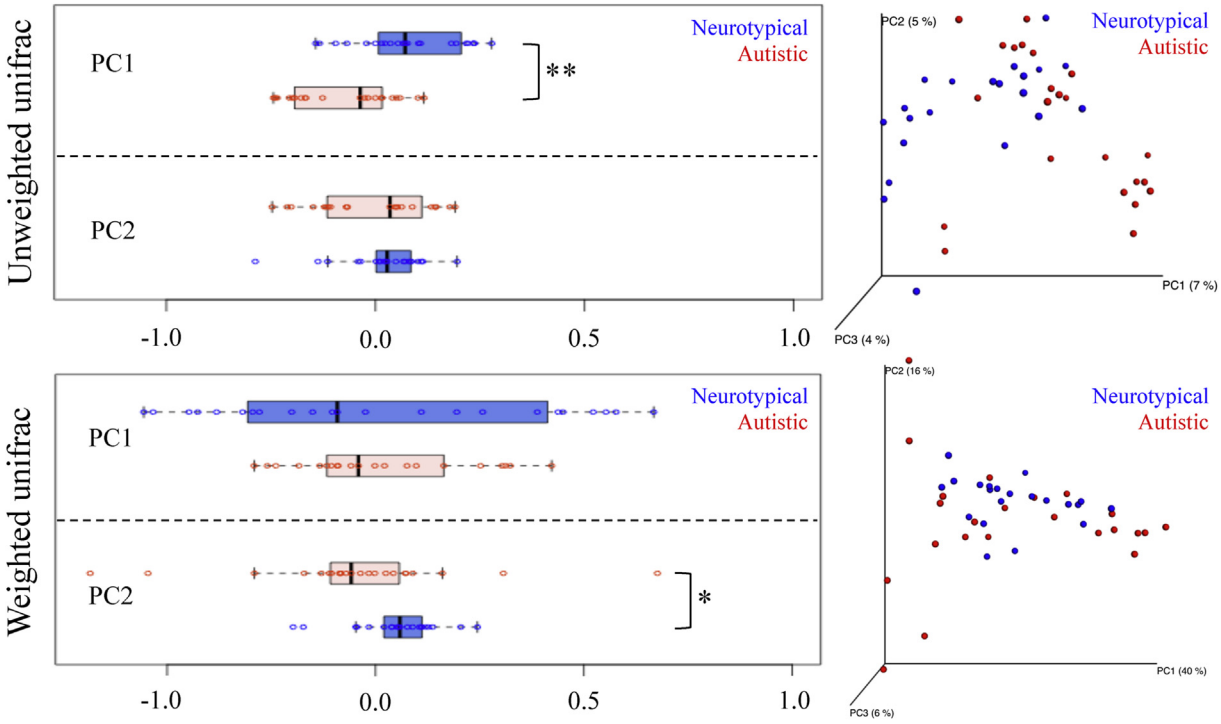

Fig. 4. Principal coordinate axis values (PC1 and PC2) and corresponding 3-dimensional PCoA plots based on unweighted (above) and weighted (below) UniFrac analyses. * and ** indicates two-tailed Mann-Whitney *U* test *p* values less than 0.05 and 0.0005, respectively.

Table 4
Species level phylotypes significantly different after multiple testing correction.

Taxonomic assignment (family/genus/species)[a]	Two-tailed Mann-Whitney *U* test		Median[b] (25%/75%)	
	p value	adjusted *p*	Neurotypical (n=21)	ASD (n=23)
Pasteurellaceae/Haemophilus/parainfluenzae	<0.001	0.006	0.01 (<0.01/0.06)	0 (0/0)
Ruminococcaceae/Faecalibacterium/prausnitzii	<0.001	0.02	8.84 (5.62/12.97)	1.06 (0.53/3.41)

[a] Detailed information with a whole list of species level phylotypes are listed in the Dataset S1.
[b] Unit: the percentile (%) abundance from a total bacteria.

study. We also examined if the FDA model improves with age included as an additional variable, but when we included age as an additional variable, age was not in the top performing combinations producing more robust separation (of up to five variables set). Further studies to validate and improve these biomarker candidates are warranted with a larger study population, a cohort with a narrower age range, or a study with an intervention that changes the microbiome.

3.7. *Faecalibacterium and Haemophilus relative abundances were significantly lower in children with ASD.*

We also assessed the fecal microbiome in these same samples using 16S rRNA gene targeted sequencing produced by 454 titanium sequencer platform. For microbial diversity within samples (alpha diversity), we confirmed that children with ASD have significantly lower bacterial diversity compared to neurotypical children (Table 3, -23% *Observed OTUs* and −20% PD; two-tailed Mann-Whitney *U* test $p < 0.001$ for both *Observed OTUs* and PD), as documented before in our studies of two different cohorts in Arizona [7,76]. Although we confirmed our previous results in this new cohort, we note that there is general disagreement regarding alpha diversity in ASD vs. controls across studies. Previously, Finegold et al. [10] observed significantly higher alpha diversity in children with autism compared with neurotypical children, but non-autistic sibling controls also had significantly higher gut diversity than the unrelated neurotypical control. Similarly, other studies reported comparable alpha diversity between children with ASD and their neurotypical siblings [19,77], and one study found higher alpha diversity in children with ASD vs. their neurotypical siblings [14]. In mouse experiments, MIA offspring that display ASD symptoms have comparable alpha diversity when compared with control offspring [22], but a recent study demonstrated that an ASD mouse model induced by poly-inosinic:polycytidylic acid and valproic acid have lower gut bacterial diversity in their offspring when compared with control group [78].

We also performed UniFrac principal coordinate analysis (PCoA) to understand the similarity of microbial communities between samples (beta diversity). The PCoA analysis shows that children with ASD have significantly different gut microbiome from

Table 5
Genus level phylotypes presenting difference between 21 neurotypical children and 23 children with ASD (two-tailed Mann-Whitney *U* test, un-adjusted *p* values less than 0.10).

Taxonomic assignment (family/genus)[a]	Two-tailed Mann-Whitney *U* test		Median[b] (25%/75%)	
	p value	adjusted *p*	Neurotypical (n=21)	ASD (n=23)
Pasteurellaceae/Haemophilus	<0.001	0.004	0.011 (0.004/0.059)	0 (0/0)
Ruminococcaceae/Faecalibacterium	<0.001	0.021	9.229 (5.784/13.691)	1.075 (0.522/3.531)
Erysipelotrichaceae/unclassified	0.002	0.123	0.183 (0.089/0.344)	0.022 (0/0.102)
[*Barnesiellaceae*]/*Barnesiella*	0.003	0.131	0.003 (0/0.019)	0 (0/0)
Christensenellaceae/Christensenella	0.004	0.131	0.005 (0/0.016)	0 (0/0)
Prevotellaceae/Prevotella	0.006	0.131	0.384 (0.007/9.112)	0 (0/0.023)
Ruminococcaceae/Butyricicoccus	0.006	0.131	0.044 (0.017/0.145)	0.007 (0/0.034)
Lachnospiraceae/Roseburia	0.007	0.131	0.429 (0.132/1.444)	0.081 (0.028/0.265)
Ruminococcaceae/unclassified	0.007	0.131	6.142 (3.705/7.194)	2.287 (0.315/5.549)
Veillonellaceae/Megamonas	0.015	0.258	0 (0/0)	0 (0/0)
Coriobacteriaceae/Eggerthella	0.018	0.260	0 (0/0)	0 (0/0.006)
Erysipelotrichaceae/unclassified	0.019	0.260	0.016 (0.008/0.056)	0 (0/0.021)
Rikenellaceae/unclassified	0.023	0.260	0.045 (0.027/0.136)	0.347 (0.084/0.602)
Lachnospiraceae/Coprococcus	0.023	0.260	0.834 (0.424/1.917)	0.173 (0.088/0.732)
[*Tissierellaceae*]/*WAL_1855D*	0.023	0.260	0.003 (0/0.044)	0 (0/0)
Ruminococcaceae/unclassified	0.029	0.306	3.645 (1.938/6.233)	1.815 (0.594/2.696)
Clostridiaceae/02d06	0.030	0.306	0.024 (0/0.066)	0 (0/0.008)
Pasteurellaceae/unclassified	0.033	0.312	0 (0/0.008)	0 (0/0)
Lachnospiraceae/Anaerostipes	0.044	0.355	0.098 (0.015/0.184)	0.010 (0/0.136)
[*Barnesiellaceae*]/unclassified	0.059	0.385	0 (0/0.038)	0 (0/0)
Turicibacteraceae/Turicibacter	0.062	0.385	0 (0/0.019)	0 (0/0)
Verrucomicrobiaceae/Akkermansia	0.063	0.385	0.075 (0.007/2.979)	0 (0/0.140)
Bacteroidaceae;/Bacteroides	0.063	0.385	22.162 (11.495/38.692)	37.158 (19.810/62.285)
Streptococcaceae/Lactococcus	0.068	0.385	0 (0/0)	0 (0/0)
Peptostreptococcaceae/Clostridium	0.068	0.385	0 (0/0)	0 (0/0)
[*Tissierellaceae*]/*Finegoldia*	0.068	0.385	0 (0/0)	0 (0/0)
[*Tissierellaceae*]/*Parvimonas*	0.068	0.385	0 (0/0)	0 (0/0)
Streptococcaceae/Streptococcus	0.070	0.385	0.097 (0.018/0.141)	0.038 (0/0.116)
Erysipelotrichaceae/Holdemania	0.074	0.395	0.032 (0.013/0.052)	0.052 (0.027/0.129)
[*Odoribacteraceae*]/*Odoribacter*	0.095	0.481	0.149 (0/0.566)	0.008 (0/0.234)
Bacteroidaceae/unclassified	0.096	0.481	0 (0/0)	0 (0/0)

[a] Detailed information with a whole list of genus level phylotypes are listed in the Dataset S1.
[b] Unit: the percentile (%) abundance from a total bacteria.

neurotypical children (Fig. 4, two-tailed Mann-Whitney *U* test, $p < 0.001$ (unweighted UniFrac PC1 axis) and 0.02 (weighted UniFrac PC2 axis).

Using the RDP classifier, we obtained bacterial profiles at the genus/species level. Previously, we determined that relative abundances of *Prevotella* and *Coprococcus* were lower in feces of children with ASD [7]. In this study, we again found that *Prevotella* was significantly reduced and *Coprococcus* had marginally lower relative abundance in the ASD group (Table S3, one-tailed Mann-Whitney *U* test, un-adjusted $p = 0.02$ and 0.09, respectively). The consistent observations between two separate recruitments are compelling considering a heterogeneity on autism spectrum disorders [20]. We also conducted an exploratory investigation by performing Mann-Whitney *U* tests on all individual genus/species level taxa and correcting for multiple tests in order to uncover any new microbiome signature for the ASD phenotype. We found that relative abundance of phylotypes most closely related to *Faecalibacterium* (*F. prausnitzii*) and *Haemophilus* (*H. parainfluenzae*) were significantly lower in children with ASD in this cohort compared with neurotypical children (Table 4 and 5, two-tailed Mann-Whitney *U* test, adjusted $p < 0.01$ and < 0.05, respectively). *F. prausnitzii* is a butyrate producer [79] and produces a 15 kDa protein named microbial anti-inflammatory molecule [80]. The abundance of *Faecalibacterium* species was negatively correlated with the severity of irritable bowel syndrome [81]. Given that children with ASD have abnormal intestinal permeability [82,83], lower relative abundances of *F. prausnitzii* in children with ASD may be related to an increased risk of inflammation in gut epithelial cells. The finding of lower relative abundances of *H. parainfluenzae* in ASD is intriguing, as other studies suggest that higher levels would be associated with a less healthy gut. *H. parainfluenzae* is known as an opportunistic pathogen associated with respiratory infections [84,85], and was prevalent in feces of infants delivered by C-section [86] and pediatric patients with IBS [87].

We also investigated the correlation between all bacterial taxa and metabolites discussed above. GABA, lactate, and formate concentrations were related with phylotypes closely assigned to *Roseburia* and *Rikenellaceae* (Table S4 and Fig. S3), but we did not observe any statistically significant correlation after multiple testing correction.

3.8. PICRUSt analysis predicts limited bacterial metabolic link to fecal metabolite profiles

Bacterial metabolic functions were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a program that estimates metabolic function based on 16S rRNA gene sequencing data and a reference genome database. With PICRUSt, we uncovered no bacterial pathways that were significantly different between the neurotypical and ASD groups after correcting for multiple hypothesis. We also investigated whether there were significant differences in genes in the metabolic pathways associated with *p*-cresol, vitamin K, and GABA, but no genes emerged significantly different between the ASD cohort and controls (adjusted *p* values > 0.3). Isopropanol producing and degrading enzymes were not investigated, since those enzymes were not listed in the PICRUSt database. Despite continuing improvement, PICRUSt has its limitations [44]. As it relies on the reference genome databases that are publically available but incomplete, predictions need to be interpreted with some caution. Metagenomic research that allows collecting actual microbial gene catalogs is warranted to better understand any potential role of gut bacteria, especially on fecal metabolites found significant in this study.

In addition to genomic database limitations, a lack of meaningful observations between fecal metabolites and microbiome profiles using PICRUSt may be attributed to a difference in dataset types, a small sample size, and statistical tools. First, ^{1}H-NMR assay provided absolute quantity of fecal metabolites, but next-generation sequencer only generated relative abundances of individual microbial components. Quantitative real-time PCR targeting specific microbes and/or microbial functions, for example, would be suitable to better define a correlation between metabolites and specific microbes. Because of a small sample size, we employed nonparametric statistical tests (e.g., Mann-Whitney *U* test and Spearman correlation test), but we may lose some sensitivity when we converted numerical values to rank-based relative values. Due to a small sample size, these results for metabolites and their correlation with microbiome should be viewed as exploratory. Although isopropanol hold its significance, other metabolites were not significantly different between groups after multiple hypothesis correction. Thus, our preliminary findings suggest that larger studies would be worthwhile and provide guidance for those larger studies.

4. Conclusion

In summary, we obtained fecal metabolite profiles in children with ASD and neurotypical children. Notably, isopropanol concentrations were significantly higher in feces of children with ASD after multiple testing corrections. Consistent with previous studies, we observed similar trends regarding higher *p*-cresol in feces of children with ASD, whereas concentrations of GABA were relatively lower in feces of children with ASD. We also confirmed lower gut microbial diversity in children with ASD in a second Arizona cohort, and reduced relative abundances of phylotypes most closely related to *F. prausnitzii*, *P. copri*, and *H. parainfluenzae* in feces of children with ASD. Although we investigated a relatively short list of fecal metabolites, the results obtained in this study support that children with ASD have different metabolite profiles in feces and warrant a more comprehensive investigation of metabolites in larger cohorts.

Data deposition

The 16S rRNA gene sequence reads analyzed in this paper were deposited in the open-source microbiom database “Qiita” with the study ID number 11169 (https://qiita.microbio.me).

Conflicts of interest

JBA, D-WK, and RKB have pending/approved patents related to the use of fecal microbiota transplant and/or probiotics for various conditions including autism. JBA is a part-time consultant for Crestovo (a microbiome-related company).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2017.12.007.

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