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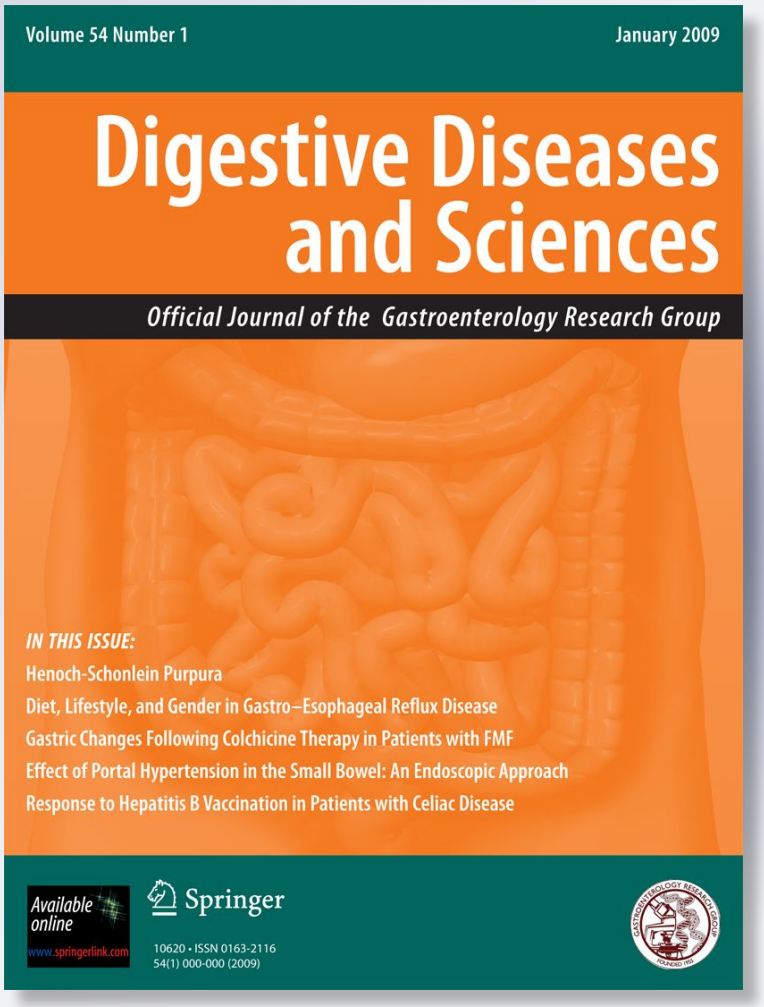

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ORIGINAL ARTICLE

Elevated Fecal Short Chain Fatty Acid and Ammonia Concentrations in Children with Autism Spectrum Disorder

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Abstract
Background and Aim Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder where a high frequency of gastrointestinal disturbance (e.g., constipation and diarrhea) is reported. As large bowel fermentation products can have beneficial or detrimental effects on health, these were measured in feces of children with and without ASD to examine whether there is an underlying disturbance in fermentation processes in the disorder.
Methods Fecal samples (48 h) were collected from children with ASD ($n = 23$), and without ASD ($n = 31$) of similar age. Concentrations of short chain fatty acids, phenols and ammonia were measured.
Results Fecal total short chain fatty acid concentrations were significantly higher in children with ASD compared to controls (136.6 ± 8.7 vs. 111.1 ± 6.6 mmol/kg). Moreover, when concentrations of fecal acetic, butyric, isobutyric, valeric, isovaleric and caproic acids were measured, all were significantly higher in children with ASD compared with controls except for caproic acid. The concentration of fecal ammonia was also significantly greater in ASD participants than controls (42.7 ± 3.3 vs. 32.3 ± 1.9 mmol/kg). Fecal phenol levels and pH did not differ between groups. Macronutrient intake, as determined from dietary records kept by caregivers, also did not differ significantly between study groups.
Conclusions Our results suggest fermentation processes or utilization of fermentation products may be altered in children with ASD compared to children without ASD.

Keywords Autism spectrum disorder · Short chain fatty acids · Phenols · Ammonia

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Introduction

Autism spectrum disorder (ASD) is a genetically determined neurodevelopmental disorder with environmental factors impacting on susceptibility [1]. Previous studies reporting the prevalence of gastrointestinal (GI) disorders in autism are conflicting [2]. An earlier study by our group found that almost half of the children with ASD that we examined had ongoing GI disturbance [3]. Genetic evidence has recently been provided supporting the link between GI dysfunction and ASD [4].

A well-balanced intestinal microbiota plays a crucial role in maintaining the GI tract and general health of the host. Altered GI microbiota has been reported in ASD

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compared with controls [5, 6]. Large bowel microbiota can impact on health through the fermentation of undigested food components and the subsequent production of a range of bioactive compounds, including short chain fatty acids (SCFA) [7]. Fermentation products other than SCFA, particularly protein fermentation by-products such as phenols, amines, ammonia and hydrogen sulfide, can be adverse to host health and toxic to the large bowel [8, 9]. Williams et al. [10] recently reported impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with ASD and GI disturbance.

It is important to know whether the concentrations of GI microbial fermentation products, such as carbohydrate and protein fermentation products, are altered in ASD and, if so, whether alterations in fermentation processes contribute to GI disturbance. Consequently, fecal concentrations of large bowel fermentation products were compared between children with and without ASD.

Methods

Participants and Sample Collection

The inclusion criteria for participants were as previously described [3]. Samples were collected from 23 children with ASD and 31 children of similar age without ASD (comprised of 22 typically developing siblings of participants with ASD and nine community controls without a family history of ASD). Participants' caregivers were required to complete three questionnaires: the first assessed the presence of GI disorders [11], the second enquired about medication (conventional and complementary) use, and the third assessed dietary intake of macro-nutrients (Cancer Council, Victoria). Samples were taken from children when they were generally in good health.

Fecal specimens were collected from participants over a 48 h period and stored at −80 °C until processing. Specimens were defrosted at room temperature and all processing was performed under anaerobic conditions. After stool mass (48 h) was recorded, fecal samples were homogenized and aliquots taken for analysis of fermentation products.

Measurement of Fermentation Products

Ammonia concentrations in fecal specimens were determined according to a modified method of Chaney et al. [12]. Fecal phenol and *p*-cresol concentrations were investigated according to the method of King et al. [13], and measurement of fecal levels of SCFA were performed as described previously by McOrist et al. [14]. Excretion was calculated as the total amount of the fermentation product present in the feces collected over 48 h (i.e., product concentration × wet weight of feces).

Statistical Analysis

Statistical analyses were conducted using SPSS for Windows™ (version 17). As the distributions of phenol and caproic acid were skewed to the left, these data were normalized by logarithmic transformation. Transformed values were used in subsequent analyses. Differences between the ASD and control groups in terms of participant characteristics were assessed using the *T* test and chi-squared test for continuous and categorical data, respectively. Any participant characteristics found to be statistically significantly different between ASD and control groups were adjusted for in all analyses of fecal fermentation products. Analysis of covariance was primarily used to determine the association between fecal fermentation product concentrations and ASD. Participant characteristics differing between ASD and control groups were controlled (adjusted) for by including the participant characteristic as a covariate. Statistical significance was deemed as $P < 0.05$ for all analyses.

Ethics Approval

Ethics approval was granted by the University of South Australia's ethics committee and adhered to the guidelines of the National Health and Medical Research Council (Australia).

Results

Characteristics of Participants and Nutrient Intake

Participants' characteristics and macro-nutrient intakes are summarized in Table 1. There were similar daily intakes of protein, sugars, starch and fiber between the children with and without ASD. The only participant characteristic that differed between the two groups was gender. Consequently, the difference in distribution of gender between the two groups was controlled for in all analyses of fermentation products by including gender as a covariate in the analysis of covariance.

Large Bowel Fermentation Products

There were significant changes in the fecal concentrations of several key bacterial fermentation products (Table 2). Fecal total SCFA concentrations were significantly higher in children with ASD (136.6 mmol/kg wet fecal weight) compared with the control group (111.1 mmol/kg wet fecal

Table 1 Participants' characteristics

Characteristic	ASD[a]	Controls	*P*-value
Number	23	31	NA
Age in months[c] (range)	123 ± 9 (37–208)	136 ± 9 (42–221)	0.345
Gender (male/female)	21/2	15/16	0.008[b]
GI disturbance (present/unpresent)	9/14	7/24	0.310
Constipation only	4	5	NA
Diarrhea only	1	1	NA
Constipation and diarrhea	4	1	NA
Nutrients[c]			
Energy (kJ/d)	7227.5 ± 466.4	7506.2 ± 467.4	0.684
Starch (g/d)	105.2 ± 7.8	102.6 ± 6.2	0.787
Protein (g/d)	77.8 ± 5.6	84.5 ± 6.9	0.478
Fat (g/d)	74.5 ± 5.8	79.3 ± 5.5	0.561
Fibre (g/d)	18.1 ± 1.5	19.1 ± 1.6	0.653
Sugar (g/d)	81.8 ± 8.1	84.0 ± 5.5	0.813
Dietary intervention			
Gluten and casein-free diet (implementation/without implementation)	3/20	0	NA
Casein-free diet only	1	0	NA
Medications (taken/not taken)	3/20	2/29	
Probiotics	2	1	NA
Antibiotics	1	1	NA

[a] Children with autism spectrum disorder

[b] Statistically significant ($P < 0.05$)

[c] The values of age and nutrients were presented as mean ± SEM

weight). Moreover, significantly elevated levels of ammonia were found in children with ASD (42.7 mmol/kg wet fecal weight) compared with controls (32.3 mmol/kg wet fecal weight). However, fecal levels of phenol and *p*-cresol did not significantly differ between children with and without ASD. The fecal concentrations of the fermentation products of each individual in the study are presented as scatter plots in Supplementary Fig. 1 and indicate which children belong to various sub-groups (i.e., children on special diets, probiotics, antibiotics, those with GI disturbance and those belonging to the different control groups). A greater spread of some minor SCFA concentrations is evident in children with ASD compared to the controls but otherwise the range of concentrations was similar between groups.

Exploration of the major and minor forms of SCFA that comprise the total SCFA concentration revealed that there was a consistent elevation in the concentration of these forms in the ASD group compared to the control group (Table 3). Specifically, there were significantly higher concentrations of acetic, propionic and butyric acids in children with ASD compared with controls. Furthermore, with the exception of caproic acid, the minor SCFA were also found to be elevated in children with ASD compared to controls (Table 3).

Generally, the bowel movement frequency of each participant was once or twice per day and there was no significant difference between children with and without ASD. The mean fecal output (g/48 h) of ASD children (168.5 ± 23.1) was not significantly different from the

Table 2 Fecal concentrations of large bowel fermentation products in ASD participants and controls

Concentration (mmol/kg wet fecal weight)	ASD[a] Mean ± SEM (range)	Controls Mean ± SEM (range)	*P*-value
Total SCFA	136.6 ± 8.7 (65.7–202.4)	111.1 ± 6.6 (59.5–192.4)	0.012[b]
Ammonia	42.7 ± 3.3 (12.9–81.6)	32.3 ± 1.9 (11.3–53.9)	0.007[b]
p-cresol	0.8 ± 0.1 (0.2–1.5)	0.8 ± 0.1 (0.3–1.7)	0.884
Phenol (10^{-3})	13.9 ± 4.4 (3.9–107.0)	8.7 ± 0.8 (3.7–18.6)	0.498

[a] Children with autism spectrum disorder

[b] Statistically significant ($P < 0.05$)

Table 3 Fecal concentrations of individual SCFA in ASD participants and controls

Concentration (mmol/kg wet fecal weight)	ASD[a] Mean ± SEM (range)	Controls Mean ± SEM (range)	*P*-value
Acetic acid	74.8 ± 5.1 (31.5–116.9)	61.7 ± 3.9 (31.2–112.5)	0.037[b]
Propionic acid	24.7 ± 1.7 (12.1–41.7)	19.7 ± 1.3 (7.4–35.0)	0.007[b]
Butyric acid	24.5 ± 1.8 (12.3–41.2)	19.5 ± 1.5 (7.8–45.5)	0.025[b]
Isobutyric acid	3.1 ± 0.3 (0.9–5.6)	2.5 ± 0.1 (1.2–4.6)	0.022[b]
Valeric acid	3.6 ± 0.3 (1.3–6.5)	2.8 ± 0.2 (0.3–5.1)	0.004[b]
Isovaleric acid	4.7 ± 0.5 (1.3–8.9)	3.9 ± 0.2 (1.5–6.9)	0.038[b]
Caproic acid	1.3 ± 0.3 (0.1–5.5)	0.7 ± 0.1 (0.1–4.1)	0.147

[a] Children with autism spectrum disorder

[b] Statistically significant ($P < 0.05$)

output of controls (156.0 ± 20.3). Fecal pH was also similar between children with and without ASD (6.8 ± 0.1 vs. 6.9 ± 0.1). Furthermore, the 48 h fecal excretion of each fermentation product examined was not significantly changed (data not shown). We also examined whether differences in concentrations of the fermentation products existed between the sibling and community controls (data not shown). Higher fecal concentrations of phenol ($P = 0.036$) and lower concentrations of *p*-cresol ($P = 0.026$) were found in siblings compared to community controls but there were no significant differences between the two control groups when total and individual SCFA, ammonia, pH and fecal output were examined. If participants were implementing special diets or using antibiotics or probiotics at the time of sampling, comparisons were also made with their data excluded from the analyses and the key findings remained unchanged (data not shown). When participants reporting a GI disturbance were excluded from analyses, there remained significant differences between ASD and controls for total SCFA, butyric acid, propionic acid, valeric acid and ammonia (data not shown). There were no significant correlations between the fecal concentrations of fermentation products and intakes of dietary macronutrients.

Discussion

We have demonstrated for the first time that fecal levels of large bowel fermentation products, particularly SCFA and ammonia, are significantly higher in children with ASD compared to controls. As these fermentation products can impact on the health of the large bowel, the observed differences may be a reason for alterations in GI health or function in children with ASD compared with children without ASD.

A notable finding of this study was the significantly higher fecal concentration of a major SCFA, propionic acid, in children with ASD. Studies in rats by MacFabe et al. [15, 16] have shown that intraventricular administration of propionic acid induced behaviors resembling ASD and reproduced the neuropathological changes reported to occur in ASD. In humans, most propionic acid is absorbed by the colon, and only 10 % of propionic acid is transported into peripheral blood under normal conditions [17]. Consequently, the magnitude of the observed increase in fecal propionic acid concentrations in ASD may not translate into clinically significant increases in blood propionic acid concentrations, but regardless warrants further investigation.

We found increased fecal concentrations of other major SCFA, namely, acetic and butyric acids as well as total SCFA in children with ASD. Similar differences in total SCFA and acetic acid concentrations have been reported by Tjellstrom et al. [18] in children with celiac disease compared with controls. Recent studies suggest that SCFA, in particular acetic acid, could have a role in gut epithelial barrier function [19, 20]. Increased intestinal permeability has been reported in ASD [21, 22] and this could be related to changes in fecal production of acetic acid or other SCFA. Consistent with the study of Tjellstrom et al. in celiac disease [18], we also found higher concentrations of the minor SCFAs, isobutyric and isovaleric acids, in ASD compared to controls. We also found that the spread of concentrations of the minor SCFAs among children appeared highest in the children with ASD and the spread of these SCFAs in sibling controls appeared intermediate between ASD and community controls. This may suggest different sub-types within the current ASD group. Intermediate effects in siblings compared with ASD and control participants (without family history of ASD) have previously been reported [3, 23] and may suggest the presence of common genetic and/or environmental influences in ASD and their siblings. Another study showed that the GI microbiota from patients with inflammatory bowel disease (IBD) produced more SCFA, especially isobutyric and

isovaleric acids, than the microbiota from healthy controls [24]. None of the participants in our study were reported as having celiac disease or IBD and there were no clear relationships between GI symptoms and fermentation products levels.

Our findings are in contrast with a recent study by Adams et al. [25] who reported lower fecal concentrations of some SCFA in children with ASD compared with controls. The differences in these findings may relate to the use of dietary supplements that can influence activity of the gut microbiota and fermentation processes. Adams et al. speculated that the lower SCFA levels may be due to probiotic usage by participants in their study. Only three participants were using probiotics during the sample collection period of our study, and exclusion of these participants did not significantly influence results.

Some protein fermentation products were also examined in this study. Fecal ammonia concentrations were significantly elevated in ASD compared with controls. High colonic levels of ammonia can damage the epithelial cells in the colon and increase gut permeability [9]. The relationship between fecal ammonia levels and gut permeability warrants further investigation. Several studies have described elevated plasma ammonia levels in children with ASD [26, 27], which may be indicative of urea cycle dysfunction. As most ammonia enters the circulation from the GI tract [28], it is plausible that the higher fecal ammonia concentrations in the ASD group translates into higher plasma and brain ammonia concentrations, also warranting further investigation.

There were no significant differences in fecal phenol or *p*-cresol concentrations between children with and without ASD. In contrast, one recent study reported high urinary *p*-cresol concentrations [29] and another study reported lower urinary *p*-cresol sulfate [30] concentrations in ASD children compared with controls. Although urinary levels of phenol and *p*-cresol may reflect gut microbial changes [31], it has been suggested that it is preferable to measure phenols in feces as urinary levels may not accurately reflect colonic production [32].

It is interesting that the differences in fermentation product concentrations were still observed when the data from children with GI disturbance in both groups were removed from analyses. GI disturbance in children with ASD is likely to be under-reported because of their communication difficulties and alterations in perception of pain which complicates how this finding is interpreted.

The mean age of our study participants was 11–12 years and all were older than 2 years. There was no significant association between age and any of the fecal fermentation product concentrations. This was expected given the colonic microbiota, and hence fermentation capacity has been described as "adult-like" in humans after the age of 2 years [33]. The gender ratio in ASD and control groups was 10:1 and 1:1, respectively, reflecting the known higher incidence of ASD in males [34]. Although the lack of a gender matched control group is a potential limitation of the present study, this difference was controlled for in all analyses of the fermentation products and is unlikely to have confounded the relationships reported.

Alterations in the populations or activities of microbiota in the GI tract, especially in the colon, could impact on the capacity of individuals to ferment dietary substrates. A recent study published by our group reported an altered GI microbiota profile, specifically low relative abundances of the mucolytic bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp., in the faeces of the same cohort of children with ASD compared with controls [5]. However, these differences in microbiota are not sufficient to explain the observed differences in fecal concentrations of SCFA and ammonia between children with and without ASD. Although altered patterns of bacterial fermentation products can be indicative of variation in nutrient intake, participants in this study had similar macronutrient intakes, suggesting dietary factors alone are unlikely to explain the observed differences in patterns of bacterial fermentation products between study groups. Further, although casein-free and/or gluten-free diets can influence fecal fermentation product concentrations, the observed significant differences between study groups were not altered when we excluded the four ASD children implementing these diets.

Fecal sample collection was taken from children when they were generally in good health, and concentrations of fecal fermentation products of participants were within the wide range that has been reported previously for apparently healthy controls [35]. However, the fecal concentrations of SCFA and ammonia in the ASD group are significantly higher than in controls, which may be indicative of alterations in processes occurring within the GI tract of children with ASD. Hence, factors which may contribute to the altered fermentation product profile require further investigation. Although most of the SCFA are utilized by the large bowel and what remains in the stool is only a small fraction of what is generated, the fecal levels are generally good indicators of SCFA production within the GI tract. However, differences in GI tissue uptake could potentially contribute to differences in levels of fecal SCFA or other bacterial products. If the higher fecal SCFA concentrations in ASD reflect greater SCFA production, then the increases may not be detrimental to the health of these individuals as SCFA have wide-ranging activities within the large bowel and are generally regarded as being beneficial [36]. Butyric acid is the primary energy source for cells lining the colon and contributes to the maintenance of colonic tissue integrity [7], whereas acetic acid has recently been shown to help counteract the toxic effects of some bacteria [37].

Fecal ammonia levels have also been shown to be positively associated with SCFA levels, which may reflect stool acidification leading to formation of ammonium and a decreased absorption by the colon [35]. However, this mechanism may not completely explain the increase in ammonia in children with ASD in this study, since pH was not significantly lower in feces of ASD children.

Statistically significant differences were detected for fecal SCFAs and ammonia; however, overlap was observed between groups and it is presently unclear how large concentration differences in fecal fermentation products must be for important biological and/or clinical manifestations.

In conclusion, we found significantly higher fecal concentrations of SCFA and ammonia in children with ASD compared with controls. While the higher concentrations may be related to their altered GI function and overall health status, they could also be due to differences in large bowel uptake and/or enteric microbiota profiles. Therefore, the underlying causes of the observed higher levels of fecal SCFA and ammonia require further investigation.

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Conflict of interest None.

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