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Low Relative Abundances of the Mucolytic Bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp. in Feces of Children with Autism▽†

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Gastrointestinal disturbance is frequently reported for individuals with autism. We used quantitative real-time PCR analysis to quantify fecal bacteria that could influence gastrointestinal health in children with and without autism. Lower relative abundances of *Bifidobacteria* species and the mucolytic bacterium *Akkermansia muciniphila* were found in children with autism, the latter suggesting mucus barrier changes.

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder where gastrointestinal (GI) disturbance is commonly reported (11). Evidence is emerging that the profiles of the GI microbiota (8, 9, 16, 22) and fermentation products (2, 28) in individuals with ASD are different from those of the general population. Finegold et al. (9) have reported a relationship between regressive autism and altered GI microbiota. Indeed, the modulation of intestinal microbiota in children with ASD through the use of antibiotics (19) and probiotics such as *Lactobacillus plantarum* WCSF1 (17) has been shown to improve behavior and bowel health outcomes. In this study, various GI bacteria, including *Clostridium* spp., members of the *Bacteroides fragilis* group, *Akkermansia muciniphila*, and *Prevotella* species, which are emerging as important markers of GI health, were examined in children with ASD, their siblings, and community controls. We also investigated whether correlations exist between GI microbial abundances and the presence or absence of caregiver-reported functional GI disorders (FGIDs) in children with ASD.

The inclusion criteria for participants were as previously described (26). Briefly, fecal samples were collected from children with ASD ($n = 23$), recruited through Autism SA, who were diagnosed by a multidisciplinary team using the childhood autism rating scale (20) and/or the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) (3). The ASD participants were diagnosed with autistic disorder ($n = 17$) and Asperger's syndrome ($n = 6$), and all but three participants were reported to have a regressive form of autism. Children who met the criteria for ASD but presented with a comorbid diagnosis of a chromosomal abnormality were excluded from the study. We also recruited 22 typically developing siblings (SIB) of the ASD cohort as well as 9 unrelated community controls (CON) without a family history of autism as two different control groups. The CON participants were eligible to participate if they did not have a sibling or first cousin with ASD. Participants' caregivers were required to complete a functional gastrointestinal disorder (FGID) questionnaire (21) and a questionnaire that investigated medication use.

Fresh fecal specimens were collected from participants over a 48-h period to exclude day-to-day variability. Each bowel movement was collected in a separate bag and frozen immediately in a portable freezer and stored at −20°C. Specimens were transported to the laboratory in freezers and stored at −80°C until they were processed. Specimens were defrosted at room temperature, and all processing was performed under anaerobic conditions (Bactron IV anaerobic chamber; Sheldon). The mass of each stool sample was recorded prior to being combined with individual participants' other stool samples collected in the 48-h sampling period. Fecal samples were homogenized, and fecal aliquots were taken for DNA extraction and analysis.

DNA was extracted from 0.25 g fecal matter using a repeat bead beating plus column method (29). The primers and optimized quantitative real-time PCR (qPCR) conditions used are summarized in Table S1 in the supplemental material. All qPCR analysis was performed on a CFX 384TM real-time PCR detection system (Bio-Rad, Hercules, CA) in triplicate and a total volume of 10 μl. Each reaction mixture consisted of 4 μl (1 ng/μl) DNA template and 6 μl PCR mixture containing 5 μl SsoFast EvaGreen Supermix, 0.5 μl bovine serum albumin, forward and reverse primers, and PCR-grade water (Sigma-Aldrich, St. Louis, MO). The qPCR cycling conditions were as follows: hot start at 98°C for 3 min, followed by 35 cycles of two-step qPCR with denaturing at 98°C for 15 s, using the annealing/elongation times and temperatures shown in Table S1 in the supplemental material. This was followed by fluorescence acquisition after each cycle. A final melt curve analysis was performed after completion of all cycles, with fluorescence acquired at 0.5°C intervals between 55 and 95°C to verify specificity of amplification. A series of eight 10-fold

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TABLE 1. Characteristics of participants[a]

Status of participant	Total no. of participants	Age (mo) [mean ± SEM (range)]	Gender (male/female)	No. of participants with/without FGIDs	No. of participants with:		No. of participants using:	
					Gluten- and casein-free diet	Casein-free diet	Antibiotics	Probiotics
ASD	23	123 ± 9 (37–208)	21/2	9/14	3	1	1	2
SIB	22	144 ± 12 (55–221)	11/11	6/16			1	1
CON	9	114 ± 15 (42–182)	4/5	1/8				

[a] ASD, autism spectrum disorder; SIB, typically developing sibling; CON, independent control; FGIDs, functional gastrointestinal disorders.

dilutions of a plasmid construct containing the target amplicon were analyzed in parallel with DNA samples for estimation of absolute abundance and PCR efficiency. Data were analyzed with Bio-Rad CFX Manager software (version 1.5) for absolute quantities (see Table S2 in the supplemental material). Relative abundances of bacterial groups were analyzed with qbase+ (Biogazelle, Ghent, Belgium) (10, 23). Data were normalized by $\log_{10}$ transformation before statistical analyses were conducted, using SPSS for Windows (version 17; SPSS Inc., Chicago, IL). A P value of less than 0.05 was considered statistically significant.

Analysis of participants' characteristics showed a higher incidence of FGIDs in children with ASD than in CON participants, whereas the incidence of FGIDs in the SIB participants was intermediate between the ASD and CON participants (Table 1). Of the 23 children with ASD, nine had GI problems, and of those, four had both constipation and diarrhea, four had constipation only, and one had diarrhea only. Of the siblings, one had both constipation and diarrhea, one had diarrhea only, and four had constipation only. One of the children in the community control group was experiencing constipation.

A lower relative abundance of *Bifidobacterium* spp. in ASD participants than in CON ($P = 0.006$) and SIB ($P = 0.032$) participants was observed. Abundance of *A. muciniphila* was decreased in ASD participants relative to that in CON participants ($P = 0.029$) and decreased in SIB participants relative to that in CON participants ($P = 0.031$). Moreover, there were elevated relative numbers of the *B. fragilis* group in ASD children experiencing FGIDs compared to those without FGIDs ($P = 0.019$). No other bacterial targets differed in abundance between study groups (Table 2).

The lower relative abundance of *Bifidobacterium* spp. in ASD participants relative to controls (SIB and CON) in our study is consistent with the recent findings of Adams et al. (1). In contrast, another study reported similar levels of *Bifidobacterium* spp. in children with and without ASD (16). ASD participants in the latter study had taken several courses of antibiotics (90%) and/or probiotics (53%) prior to entering the study, which may have influenced composition of the GI microbiota. Notably, only one of our ASD participants received antibiotics during the week prior to the sampling period. Furthermore, none of the SIB or CON participants received antibiotics immediately prior to or during the sampling period. In addition, two of our ASD participants were taking probiotics during the sample collection period. Further investigation of the relationship between *Bifidobacterium* spp., antibiotics, probiotics, and ASD is warranted. Using the correct species of *Bifidobacterium* spp. will be important; e.g., *Bifidobacterium longum* Ncc3001 has been shown to improve anxiety in mice via the vagal nerve (15).

A. muciniphila is a mucin-degrading bacterium present in abundance in the guts of healthy adults, but numbers are reduced in patients with Crohn's disease or ulcerative colitis and in the elderly (4, 14, 18). This indicates that *A. muciniphila* could be an important marker for gut health. A thinner mucus layer is often present in patients with ulcerative colitis than in controls (5), which probably represents less substrate for mucin-degrading bacteria and hence lower numbers in the feces. Therefore, our finding of a lower abundance of *A. muciniphila* in ASD children and their siblings may indicate a thinner GI mucus barrier in ASD children than in the CON participants. These results could represent indirect evidence of impaired gut permeability in children with ASD (6, 7). A previous study (6) has indicated that there may be increased gut permeability in ASD children and their first-degree relatives. Our finding of decreases in *A. muciniphila* in both ASD children and their siblings could support this hypothesis. Although we have suggested that a lower relative abundance of *A. muciniphila* may represent altered mucus turnover, we have insufficient knowledge to determine if this actually represents a beneficial or detrimental difference. Other bacteria not measured in this study can also degrade mucus and could also potentially contribute to altered mucus barrier function and/or perturb levels of *A. muciniphila*. Hence, analysis of numbers of a wider range

TABLE 2. Relative abundances of target bacteria[a]

Target[b]	Relative no. (mean ± SEM)[c]		
	ASD	SIB	CON
Akkermansia muciniphila	5.07 ± 1.54^{A}	4.67 ± 1.72^{A}	14.18 ± 6.21^{B}
Bacteroides fragilis group	1.25 ± 0.32	1.75 ± 0.44	2.45 ± 1.82
Bifidobacterium spp.	0.78 ± 0.18^{A}	1.37 ± 0.27^{B}	1.96 ± 0.48^{B}
Clostridium coccoides group	1.21 ± 0.17	1.01 ± 0.11	1.14 ± 0.16
Clostridium leptum group	0.98 ± 0.12	0.99 ± 0.08	1.13 ± 0.16
Clostridium cluster I	1.20 ± 0.28	0.89 ± 0.17	1.72 ± 0.49
Clostridium difficile	3.23 ± 2.82	0.45 ± 0.07	0.32 ± 0.06
Enterococcus spp.	1.39 ± 0.76	0.71 ± 0.25	1.47 ± 0.80
Escherichia coli	2.95 ± 1.48	7.40 ± 5.55	3.22 ± 1.21
Faecalibacterium prausnitzii	1.18 ± 0.17	1.13 ± 0.13	0.73 ± 0.15
Lactobacillus group	0.81 ± 0.29	0.99 ± 0.43	0.72 ± 0.27
Prevotella group	1.04 ± 0.33	1.49 ± 0.43	0.44 ± 0.13
SRB_*aps*	0.57 ± 0.09	0.79 ± 0.19	0.33 ± 0.07
SRB_*dsr*	0.59 ± 0.12	0.70 ± 0.19	0.70 ± 0.11

[a] Relative abundances were calculated using qbase+ (10, 23).

[b] SRB, sulfate-reducing bacteria; *aps*, adenosine-5′-phosphosulfate reductase gene; *dsr*, α subunit of the dissimilatory sulfite reductase gene (*dsrA*).

[c] Superscript A or B next to data in the same line indicates that significant differences exist between participant groups ($P < 0.05$).

of mucus-degrading bacteria in feces of children with ASD in future studies would be informative.

Species within the *B. fragilis* group have beneficial effects on host health, while others cause infections with significant morbidity and mortality (27). As higher numbers of the *B. fragilis* group were found in ASD children with reported FGIDs, it is possible that some species belonging to the *B. fragilis* group are responsible for GI pathology in children with autism. Future analyses that target specific members of the *B. fragilis* group will shed further light on the species involved.

In contrast to results from previous studies (16, 22), in this study similar levels of bacteria from *Clostridium* cluster I were found in all participants. A higher abundance of members of the *Clostridium histolyticum* group (*Clostridium* cluster I plus cluster II) was reported in children with ASD by Parracho et al. (16). However, most participants (76%) in their study had diarrhea, and 66% of participants were implementing gluten-free and/or casein-free diets. Also, the results of Parracho et al. were based on a single fecal sample from each participant. We collected samples over a period of 48 h, which provides a better representation of the GI microbial population and eliminates diurnal variations. The most common FGID symptom in ASD in our cohort was constipation, and only four children were implementing a gluten-free and/or casein-free diet. The study by Song et al. (22) showed significantly higher numbers of *Clostridium* cluster I and *Clostridium bolteae* bacteria in fecal specimens of children with ASD than that in controls, but they provided only limited information regarding participants or sample collection methodology, both of which are needed to understand points of difference with the current study. Thus, the different findings regarding the abundance of *Clostridium* species may relate to diverse presentation of FGIDs and various dietary interventions in the study cohorts. In addition, numbers of targeted bacteria such as *Faecalibacterium prausnitzii*, members of the *Clostridium leptum* group, and members of the *Clostridium coccoides* group, which cover many of the primary butyrate producers and are beneficial for gut health, did not significantly differ among the study groups.

Previous studies in rats by MacFabe et al. have shown that intraventricular administration of propionate induces behaviors resembling autism (e.g., repetitive dystonic behaviors, retropulsion, seizures, and social avoidance) (12, 13). We have also reported increased fecal propionate concentrations in ASD children compared with that in controls in the same fecal samples (25). However, the abundance of a key propionate-producing bacterium, *Prevotella* sp., was not significantly different between the study groups. This suggests that other untargeted bacteria, such as those from *Clostridium* cluster IX, which also includes major propionate producers (24), may be responsible for the observed differences in fecal propionate concentrations. Moreover, it is possible that the activities of the bacteria responsible for producing propionate, rather than bacterial numbers, have been altered. Other factors, such as differences in GI function that change GI transit time in ASD children, should also be considered.

In summary, the current findings of depleted populations of *A. muciniphila* and *Bifidobacterium* spp. add to our knowledge of the changes in the GI tracts of ASD children. These findings could potentially guide implementation of dietary/probiotic interventions that impact the gut microbiota and improve GI health in individuals with ASD.

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