The relative abundance of the mucolytic bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp. is lower in feces of children with autism

**Authors:** Lv Wang, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia 5001, Australia; Claus Thagaard Christophersen, Preventative Health National Research Flagship, CSIRO Food and Nutritional Sciences, Adelaide, South Australia 5001, Australia; Michael Joseph Sorich, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia 5001, Australia; Jacobus Petrus Gerber, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia 5001, Australia; Manya Therese Angley, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia 5001, Australia; Michael Allan Conlon, Preventative Health National Research Flagship, CSIRO Food and Nutritional Sciences, Adelaide, South Australia 5001, Australia.

Running title: Lower abundance of *Akkermansia muciniphila* in autism

---

1 Corresponding author: Dr. Manya Therese Angley, Sansom Institute for Health Research, University of South Australia, GPO Box 2471, Adelaide, South Australia 5001, Australia, Tel +61 8 8338 6467, Fax: +61 8 8302 2389, Email: manya.angley@unisa.edu.au.
Abstract
Gastrointestinal disturbance is frequently reported in autism. We used quantitative real-time PCR to quantify fecal bacteria that could influence gastrointestinal health in children with and without autism. Lower relative abundances of *Bifidobacteria* 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 [12]. Evidence is emerging that the profiles of the GI microbiota [9, 10, 23, 31] and fermentation products [2, 38] in ASD are different from those of the general population. Finegold et al [10] 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 [28] and probiotics, such as *Lactobacillus plantarum* WCSF1 [24] has been shown to improve behavior and bowel health outcomes. In this study, various GI bacteria, including *Clostridium* spp., *Bacteroides fragilis* group, *Akkermansia muciniphila* and *Prevotella*, 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 [35]. Briefly, fecal samples were collected from children with ASD (n = 23) recruited through Autism SA who were diagnosed by a multi-disciplinary team using the Childhood Autism Rating Scale [29] 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 co-morbid 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 [30] 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 in the freezers to the laboratory and stored at -80 °C until processing. Specimens were defrosted at room temperature and all processing was performed under anaerobic conditions (Bactron IV anaerobic chamber, Sheldon, USA). Each stool mass was recorded prior to being combined with individual participants’ other stools 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 of fecal matter** using a repeat bead beating plus column method [39]. The primers and optimized quantitative real-time PCR (QPCR) conditions used are summarized in the Supplemental file (Table S1). All QPCR was performed on a CFX 384TM real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) in triplicate and a total volume of 10 µL. Each reaction consisted of 4 µL (1 ng/ µL) DNA template and 6 µL of 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, USA). The QPCR cycling conditions were as follows: hot-start 98 °C for 3 min followed by 35 cycles of two-step QPCR with denaturing at 98 °C for 15 s and annealing/elongation time and temperature as given in Table S1. 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. An 8-series of 10-fold dilutions of a plasmid construct containing the target amplicon was 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 (Table S2). Relative abundance of bacterial groups was analyzed with qBase’ (Biogazelle, Ghent, Belgium) [11, 32]. Data were normalised by log10 transformation before statistical analyses were conducted using SPSS for Windows™ (version 17, SPSS Inc., Chicago, IL, USA). 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 compared with 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 community controls was experiencing constipation.

A lower relative abundance of *Bifidobacterium* spp. in ASD compared with CON (*p* = 0.006) and SIB participants (*p* = 0.032) was observed. Abundance of *A. muciniphila* was decreased in ASD relative to CON participants (*p* = 0.029) and in SIB compared with 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 [23]. 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. Further, none of the SIB or CON participants received antibiotics immediately prior 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 shown to improve anxiety in mice via the vagal nerve [22].

A. muciniphila is a mucin-degrading bacterium present in abundance in the gut of healthy adults but numbers are reduced in patients with Crohn’s disease, ulcerative colitis and the elderly [5, 20, 26]. 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 compared with controls [6] 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 compared to CON participants. These results could represent indirect evidence of impaired gut permeability in children with ASD [7, 8]. A previous study [8] has indicated there may be increased gut permeability in ASD and their first-degree relatives. Our findings of decreases in A. muciniphila in both ASD 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 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 [37]. 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 autism. Future analysis that target specific members of the *B. fragilis* group will shed further light on the species involved.

In contrast to previous studies [23, 31], similar levels of *Clostridium cluster I* were found in all participants in this study. A higher abundance of *C. histolyticum* group (*Clostridium cluster I* plus *cluster II*) was reported in children with ASD by Parracho et al. [23]. However, most participants (76%) in their study had diarrhea and 66% of participants were implementing gluten-free and/or casein-free diets. Also, Parracho et al.’s results were based on a single fecal sample from each participant. We collected 48 h samples, 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 4 were implementing a gluten-free and/or casein-free diet. Song et al.’s study [31] showed significantly higher numbers of *Clostridium cluster I* and *C. bolteae* in fecal specimens of children with ASD compared with controls, but they only provided 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 *Clostridia* may relate to diverse presentation of FGIDs and varying dietary interventions in the study cohorts. In addition, numbers of targeted bacteria such as the *Faecalibacterium prausnitzii*, *C. leptum* group and *C. 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 behaviours resembling autism (e.g. repetitive dystonic behaviours, retropulsion, seizures and social avoidance) [17, 18]. We have also reported increased fecal propionate concentrations in ASD compared with controls in the same fecal samples [34]. However, the abundance of key propionate-producing bacteria, Prevotella, was not significantly different between the study groups. This suggests that other untargeted bacteria, such as C. cluster IX, which also include major propionate producers [33], 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 have been altered rather than bacterial numbers. Other factors, such as differences in GI function that change GI transit time in ASD, 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 tract of ASD children. These findings could potentially guide implementation of dietary/probiotic interventions that impact on the gut microbiota and improve GI health in individuals with ASD.

This research was funded by The Australian Rotary Health Research Fund. We wish to express our gratitude to the participating children and their parents. We would also like to acknowledge Dr Richard Couper, paediatric gastroenterologist, for medical advice; Mrs. Rosalind Miller for statistical analysis assistance as well as Ms. Corinna Bennett, Ms. Emma Watson and Ms. Kerry Nyland for technical assistance.
References


adherent to gastrointestinal mucosa by real-time PCR. J Clin Microbiol, 40:4423-4427.


### Table 1: Characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Age (Months) Mean ± S.E.M (Range)</th>
<th>Gender Male / Female</th>
<th>FGIDs (Yes / No)</th>
<th>Diet</th>
<th>Medicine/ Supplements use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASD</td>
<td>23</td>
<td>123 ± 9 (37-208)</td>
<td>21 / 2</td>
<td>9 / 14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>SIB</td>
<td>22</td>
<td>144 ± 12 (55-221)</td>
<td>11 / 11</td>
<td>6 / 16</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>CON</td>
<td>9</td>
<td>114 ± 15 (42-182)</td>
<td>4 / 5</td>
<td>1 / 8</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

ASD, autism spectrum disorder; SIB, typically developing siblings; CON, independent controls; S.E.M., standard error of the mean; FGID, functional gastrointestinal disorder.
Table 2: The relative abundances of target bacteria*

<table>
<thead>
<tr>
<th>Target</th>
<th>Relative numbers</th>
<th>Mean ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASD</td>
<td>SIB</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>5.07 ± 1.54</td>
<td>4.67 ± 1.72</td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td>1.25 ± 0.32</td>
<td>1.75 ± 0.44</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>0.78 ± 0.18</td>
<td>1.37 ± 0.27</td>
</tr>
<tr>
<td>Clostridium cocoides group</td>
<td>1.21 ± 0.17</td>
<td>1.01 ± 0.11</td>
</tr>
<tr>
<td>Clostridium leptum group</td>
<td>0.98 ± 0.12</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>1.20 ± 0.28</td>
<td>0.89 ± 0.17</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>3.23 ± 2.82</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>1.39 ± 0.76</td>
<td>0.71 ± 0.25</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2.95 ± 1.48</td>
<td>7.40 ± 5.55</td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii</td>
<td>1.18 ± 0.17</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>Lactobacillus group</td>
<td>0.81 ± 0.29</td>
<td>0.99 ± 0.43</td>
</tr>
<tr>
<td>Prevotella group</td>
<td>1.04 ± 0.33</td>
<td>1.49 ± 0.43</td>
</tr>
</tbody>
</table>
| SRB1
|               | 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  |

Total bacteria -- -- --

* Relative abundances calculated using qBase⁺ [11, 32]

1, Sulphate-reducing bacteria; 2, Adenosine-5-phosphosulfate reduce gene; 3, Dissimilatory sulfate reductase gene.

When * and † appear next to data in the same line, it is indicative that significant differences exist between participant groups ( P < 0.05).