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# Altered Gut Microbiota as Potential Biomarkers for Autism Spectrum Disorder in Early Childhood

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**Abstract—Gastrointestinal (GI) disorders are widely recorded in autism spectrum disorder (ASD), and ASD with GI symptoms is a vital subtype of this disease. Growing evidence suggests altered gut microbiota biomarkers in ASD, but little is known about the gut microbiota of individuals with ASD with GI Symptoms, particularly in early childhood. In our study, the gut microbiota of 36 individuals with ASD along with GI symptoms and 40 typically developing (TD) children were compared using 16S rRNA gene sequencing. The microbial diversity and composition were found to differ between the two groups. Compared to TD, the gut microbiota of ASD patients with GI symptoms exhibited decreased alpha diversity and depletion of butyrate-producing bacteria (e.g., *Faecalibacterium* and *Coprococcus*). In addition, microbial functional analysis showed abnormality in several gut metabolic models and gut brain models of ASD with GI symptoms, including short-chain fatty acid (SCFA) synthesis/degradation and neurotoxin-related p-cresol degradation, which are closely associated with ASD-related behaviors in animal models. Furthermore, we constructed a Support Vector Machine classification model, which robustly discriminated individuals with ASD and GI symptoms from TD individuals in a validation set (AUC = 0.88). Our findings provide a deep insight into the roles of the disturbed gut ecosystem in individuals with ASD and GI symptoms aged 3–6 years. Our classification model supports gut microbiota as a potential biomarker for the early identification of ASD and interventions targeting particular gut-beneficial microbiota.**

**Key words: microbiota, gastrointestinal symptoms, biomarker, early childhood, autism.**

## INTRODUCTION

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder, and its prognosis remains far from optimistic (Elder et al., 2017). Once diagnosed with ASD, caregivers experience substantial psychological pain and financial burden. Children with ASD frequently report experiencing gastrointestinal (GI) issues such as constipation, abdominal pain, and diarrhea (Leader et al., 2022), and ASD with GI symptoms may be a subtype of this disease (Yang et al., 2018). Some children's GI symptoms may not be found because of their limited verbal and communication abilities. Therefore, the prevalence of GI symptoms may be higher. If not treated in time, GI symptoms can lead to more severe ASD symptoms and other related clinical manifestations. In turn, the illness affects growth, development, nutrition, and immune status of children, creating a vicious cycle. Individuals with ASD and GI symptoms are more likely to exhibit aggressive, irritable, and self-injurious behavior (Mazefsky et al., 2014; Peters et al., 2014; Fulceri et al., 2016). These problems reduce the patients' quality of life and significantly increase the burden on caregivers (Holingue et al., 2022).

The gut microbiome, a potential mediator of risk factors for patients with ASD, has recently gained attention. Disturbances in the gut microbiome in ASD

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*Abbreviations:* ABC, Autism Behavior Checklist; ADOS, Autism Diagnostic Observation Schedule; ASD, Autism spectrum disorder; ASVs, amplicon sequence variants; ATEC, Autism Treatment Evaluation Checklist; CARS, Childhood Autism Rating Scale; GBMs, gut brain modules; GI, Gastrointestinal; GMMs, gut metabolic modules; GSI, gastrointestinal severity indices; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG orthology; LDA, Linear discriminant analysis; LEfSe, LDA effect size; PCoA, Principal coordinate analysis; SHAP, Shapley Additive exPlanations method; SCFA, short-chain fatty acid; TD, typically developing.

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have been observed in previous studies using 16S rRNA and metagenomic sequencing (Wang et al., 2019; Wan et al., 2021; Ye et al., 2021). According to various studies, altering the ecology of the gut microbiota not only improves GI symptoms but also improves the maladaptive behavior of ASD, which clarifies the critical role of the gut microbiome in ASD onset (Kang et al., 2017; Li et al., 2021; van de Wouw et al., 2021). Despite the potential implications for the pathogenesis of ASD, few studies have focused on the microbial characteristics of ASD with GI symptoms.

Three recent studies attempted to address this issue (Rose et al., 2018; Dan et al., 2020; Deng et al., 2022). One study included patients with ASD who only had constipation, and the other two focused on ASD with GI symptoms. These studies included children of different ages, and their ages varied widely. Thus, the results of these three studies are inconsistent. Gut microbiome changes from birth to adulthood (Ronan et al., 2021). Childhood, especially infancy, is a crucial period for the establishment of normal flora. According to animal and human studies, probiotics are crucial for the growth and development of children. The gut flora in early life secretes neurotransmitters that interfere with the central nervous pathways of the host, significantly affecting the later neural development of animal models (Rosenfeld, 2015). In an animal model of ASD, Li *et al*. proved that the congenital underdeveloped gut was the early driving force for the intestinal flora and hosted the neural development of ASD through oxidative stress (Li et al., 2022). The findings of Laue *et al*. support a potential link between the early childhood gut microbiome and social behavior (Laue et al., 2020). It is believed that early recognition and intervention are crucial for ASD prognosis (Lord et al., 2022), and the best time for clinical intervention in ASD is before the age of six. However, the current diagnosis of ASD relies on clinical interviews. Therefore, identifying novel molecular biomarkers for the early diagnosis of ASD, especially in children with ASD and GI symptoms (a vital subtype of this disease), is of enormous clinical importance.

To answer this question, we conducted a cross-sectional study using 16S rRNA gene sequencing of stool samples from individuals with ASD ($n = 36$) and typically developing (TD) children ($n = 40$). We tried to identify ASD with GI symptom-related microbial signatures at an early age and constructed a discriminative microbial model that could be validated in a new set.

## EXPERIMENTAL PROCEDURES

### Participants

Children with ASD in this study visited the Beijing Anding Hospital from March–September 2022. They were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (5th Edition) and the Autism Diagnostic Observation Schedule (1st Edition, ADOS-1) (Gotham et al., 2007). We evaluated GI problems using six gastrointestinal severity indices (6-GSI) (Schneider et al., 2006). All children with autism scored $> 30$ on the Childhood Autism Rating Scale (CARS), and at least one symptom of their 6-GSI score was two points. TD children without GI symptoms were recruited from kindergartens as controls. The children with ASD and TD both came from Beijing and had similar regional eating habits, and the two groups were matched in terms of gender and age. All subjects were between 3–6 years of age and completed tests for physical, mental, and behavioral health. The exclusion criteria included diseases such as nervous system disorders, schizophrenia, bipolar disorder, depressive disorder, intellectual disability, and severe physical disease. The participants' information about clinical symptoms, GI symptoms, past disease history, and medication history were gathered during sampling. All participants' parents completed self-rating scales, such as the Autism Behavior Checklist (ABC), self-designed diet questionnaire, and the Zarit Burden Interview (Lu et al., 2009). Moreover, trained child neuropsychologists assessed all children with ASD using the CARS and Autism Treatment Evaluation Checklist (ATEC) (Rimland and Edelson, 1999). The ATEC is a comprehensive evaluation tool for ASD. None of the participants had taken antibiotics, probiotics, or prebiotics in the month before fecal collection.

### Specimen collection

Feces were collected in hospitals or at home as instructed, and transported at low temperatures immediately after collection. Frozen feces were transported to the Beijing Anding Hospital on dry ice, and the specimens were divided into four portions of 200 mg each and stored at −80 °C until extraction.

### DNA Extraction, PCR Amplification, and 16S rRNA sequencing

Approximately 100 mg of fecal sample was used to extract the bacteria using a DNA Kit (Omega Bio-tek, GA, U.S.), and a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) was used to measure DNA concentration and purity. Using an ABI GeneAmp® 9700 PCR thermal cycler (ABI, CA, USA) and the primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), the hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified. The equipment used, the manufacturer's instructions, and the comprehensive PCR procedure were the same as those of Zheng et al. (2020), carried out by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

### Bioinformatic analysis

Raw sequence reads were quality filtered, truncated, and then used to infer amplicon sequence variants (ASVs) using **DADA2** (Callahan et al., 2016). Here, reads after quality control were merged and clustered into ASVs after chimeric sequence removal using the 'consensus' strategy implemented in **DADA2**. Each ASV was then assigned a taxonomy using a naive Bayes classifier trained using the SILVA database (v138). An approximate maximum-likelihood phylogenetic tree of ASV sequences was constructed using FastTree with multiple-alignment results from the **DECIPHER** package (Firth et al., 2009). The ASV abundance table, taxonomic classification, phylogenetic tree, and metadata were organized into a phyloseq object for downstream statistical analysis using the **phyloseq** package (McMurdie and Holmes, 2013).

ASV abundance was aggregated at the genus level for further analysis. After being rarefied to an equal depth, the genus alpha diversity (observed and Shannon index) was estimated using the *estimate_richness* function in the **phyloseq** package. Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity was used to analyze the differences in microbial composition. We also used **Dirichlet multinomial mixtures** to infer the optimal number of community types (Holmes et al., 2012).

Functional annotation was performed using **PICRUSt2** (Douglas et al., 2020) with default parameters, and the predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) abundance was aggregated into gut metabolic modules (GMMs) and gut brain modules (GBMs) using **omixer-rpmR** (Darzi et al., 2016). GBM corresponds to functional modules of the gut microbiome that have the potential to produce or degrade neuroactive compounds (Valles-Colomer et al., 2019).

### Network analysis

Microbial co-abundance networks were generated in the ASD and TD groups. **SparCC**, implemented in **FastSpar**, was used to calculate pairwise correlations using genus count data as the input. Permuted *P* values were calculated, and significant correlations were determined using a cutoff of $p < 0.01$. The resulting networks were visualized via **ggraph** (Si et al., 2022).

### Statistical analysis

Using the *adonis2* function in the vegan package in R, permutational multivariate analysis of variance was performed to evaluate the microbiota composition differences based on Bray–Curtis dissimilarity (Anderson, 2017). Linear discriminant analysis (LDA) effect size (LEfSe) was used to compare taxonomic differences between the groups (Paulson et al., 2013), with absolute LDA scores > 2 indicating significantly enriched taxa. The non-parametric Mann–Whitney U test was used to examine the differences in diversity indices and predicted functions between the two groups. All statistical analyses were performed using R (version 4.2) (Parnell and Jackson, 2013).

## RESULTS

### Subject characteristics

Thirty-six children clinically diagnosed with ASD (average age, 3.92 + /-1.08; gender ratio, male: female 27:9) were enrolled from March–September 2022. Forty age- and sex-matched TD children (average age 4.30 + /-1.16; gender ratio, male: female 31:9) were also enlisted from a kindergarten (Table 1). The participants' basic information, including their parents' age at birth, delivery mode, family economic status, diet structure, and family burden, were recorded. Among the 36 ASD patients, 22 had constipation and the other 14 had diarrhea, flatulence, or other GI symptoms. In addition, we collected information on the self-rating and physician rating scales of the children with ASD (Table 1).

In this group, we observed that parents of children with autism had significantly higher educational attainment ($P_{father}$ = 8.86e−04 and $P_{mother}$ = 1.06e−03) and childbearing age ($P_{father}$ = 3.30e−03 and $P_{mother}$ = 4.80e−04) than parents of children without autism. However, the two groups had no significant differences in birth patterns (cesarean section or vaginal birth). Children with ASD were more likely to have problems during pregnancy (*P* = 4.25e−02) and at birth (*P* = 2.59e−03). Regarding diet, children with ASD were more picky eaters than the TD group, and the difference was statistically significant (*P* = 4.28e−07). Dietary fiber intake was significantly lower in the ASD group than in the TD group (*P* = 1.91e−03). In addition, the family burden of children with ASD was considerably higher than that of TD children (*P* = 9.17e−14).

We conducted Spearman's correlation analysis on the phenotypic information of children with ASD, and the results are shown in Fig. 1. We found that the education level and childbearing age of the fathers of children with ASD were significantly positively correlated with the education level (*P* = 1.50e−06) and childbearing age (*P* = 1.99e−06) of the mothers. There was a significant positive association between the birth mode of cesarean section and dietary fiber intake in children with ASD (*P* = 1.30e−04). The CARS score was closely correlated with the ADOS (*P* = 2.15e−05) and ATEC scores (*P* = 0.37e−04). Simultaneously, the ABC score was significantly positively correlated with the total ATEC score (*P* = 2.48e−07). Among the ATEC subscales, ATEC_com and ATEC_cog were closely related (*P* = 2.56e−07).

### Microbiota changes in children with ASD and GI symptoms

A total of 4,541,314 valid sequencing reads (50,459 for each sample, on average) were obtained from 76 samples. After processing using the DADA2 pipeline, 1801 ASVs were obtained. The rarefaction curve showed that all samples reached high sampling coverage, indicating that the sequencing depth was sufficient to investigate the fecal flora (Fig. S1). As shown in Fig. 2(**A**), 409 ASVs were shared between the two groups.

The alpha diversity between the two groups was compared at the genus level. The results revealed that the diversity (both richness and Shannon index) in the ASD group was much lower than that in the TD group (Wilcoxon rank sum test, Fig. 2(**B**)). The similarity of the microbial communities between the two cohorts was

**Table 1.** Characteristics of the study participants

| Items | ASD ($n$ = 36) | TD ($n$ = 40) | $P$ value |
|---|---|---|---|
| **Sex** | | | 1e + 00 |
| male | 27 | 31 | |
| female | 9 | 9 | |
| **Age (mean ± sd)** | | | |
| Child | 3.92 ± 1.08 | 4.30 ± 1.16 | 1.40e−01 |
| Father_at_birth | 32.00 ± 5.22 | 28.40 ± 3.47 | 8.86e−04 |
| Mother_at_birth | 30.86 ± 4.13 | 27.85 ± 3.48 | 1.06e−03 |
| **Education,median (IQR)** | | | |
| Father | 4.00(4.00,5.00) | 4.00(2.00,4.25) | 3.30e−03 |
| Mother | 4.00(4.00,4.00) | 4.00(2.00,4.00) | 4.80e−04 |
| **Birth mode** | | | 2.60e−01 |
| cesarean | 20 | 16 | |
| normal | 16 | 24 | |
| **Problem during pregnancy** | | | 4.25e−02 |
| Yes | 7 | 1 | |
| No | 29 | 39 | |
| **Problem at birth** | | | 2.59e−03 |
| Yes | 9 | 0 | |
| No | 27 | 40 | |
| **Disease history** | | | 2.03e−01 |
| Yes | 33 | 40 | |
| No | 3 | 0 | |
| **Picky eater** | | | 4.28e−07 |
| Yes | 26 | 5 | |
| No | 10 | 35 | |
| **Diet, median (IQR)** | | | |
| High fat | 3.00(2.00,3.00) | 3.00(2.00,3.00) | 6.08e−01 |
| High salt | 2.50(2.00,3.00) | 3.00(2.00,3.00) | 9.67e−05 |
| High fiber | 3.00(1.00,3.00) | 3.00(3.00,4.00) | 1.91e−03 |
| High protein | 3.00(2.00,4.00) | 4.00(3.00,4.00) | 1.20e−03 |
| High sugar | 3.00(2.00,3.25) | 2.00(2.00,2.00) | 2.12e−01 |
| Drinks | 1.00(1.00,2.00) | 2.00(2.00,2.00) | 2.09e−01 |
| Salty snacks | 2.00(1.00,2.00) | 2.00(2.00,2.00) | 3.17e−01 |
| Vitamin | 1.00(1.00,2.00) | 1.00(1.00,1.00) | 3.64e−01 |
| **ASD score (mean ± sd)** | | | |
| CARS | 38.94 ± 4.41 | 15.00 ± 0.00 | |
| ABC | 64.86 ± 17.00 | 1.52 ± 2.31 | |
| ADOS | 17.17 ± 2.66 | NA | |
| ATEC | 67.67 ± 21.54 | NA | |
| Family burden | 41.11 ± 19.24 | 6.12 ± 3.78 | 9.17e−14 |

Father_at_birth, father's age at birth; Mother_at_birth, mother's age at birth.
IQR, interquartile range; CARS, Children's Autism Rating Scale.
Birth mode, mode of birth; Disease history, past medical history.
ABC, Autism Behavior Checklist.
ADOS, Autism Diagnostic Observation Schedule.
ATEC, Autism Treatment Evaluation Checklist.

examined using PCoA based on the Bray–Curtis distance. The ASD and TD samples were clearly separated on the PCoA plot (Fig. 2(**C**)). The Bray–Curtis distance ($R^2$ = 0.025, $P$ = 0.01) and UniFrac distance ($R^2$ = 0.028, $P$ = 0.005) (Fig. S2) in the permutational multivariate analysis of variance confirmed that there was a significant difference in the microfloral composition between the two groups.

LEfSe results indicate the differences in phylogenetic distributions of the microbiota of ASD and healthy children at all taxonomic levels (Fig. 2(**D**)). The histogram of LDA scores indicates that the relative abundance of Proteobacteria is much higher in ASD. At the genus level, *Enterococcus*, *Sellimonas*, and *Eggerthella lenta* were enriched in the ASD group. The TD group was characterized by a higher abundance of *Faecalibacterium* and *Coprococcus* at the genus level and *Faecalibacterium prausnitzii, Bifidobacterium bifidum, Coprococcus comes, and Coprococcus catus* at the species level (Fig. 2(**E**)). PERMANOVA analysis results showed no differences between boys and girls in our study (R2 = 0.0053, $P$ = 0.83).

Enterotyping, proposed as a valuable method to stratify human gut microbiomes, is associated with human diseases (Arumugam et al., 2011). We performed enterotyping using Dirichlet multinomial clustering based on the count data at the genus level (Fig. S3). This approach uncovered an optional stratification of two

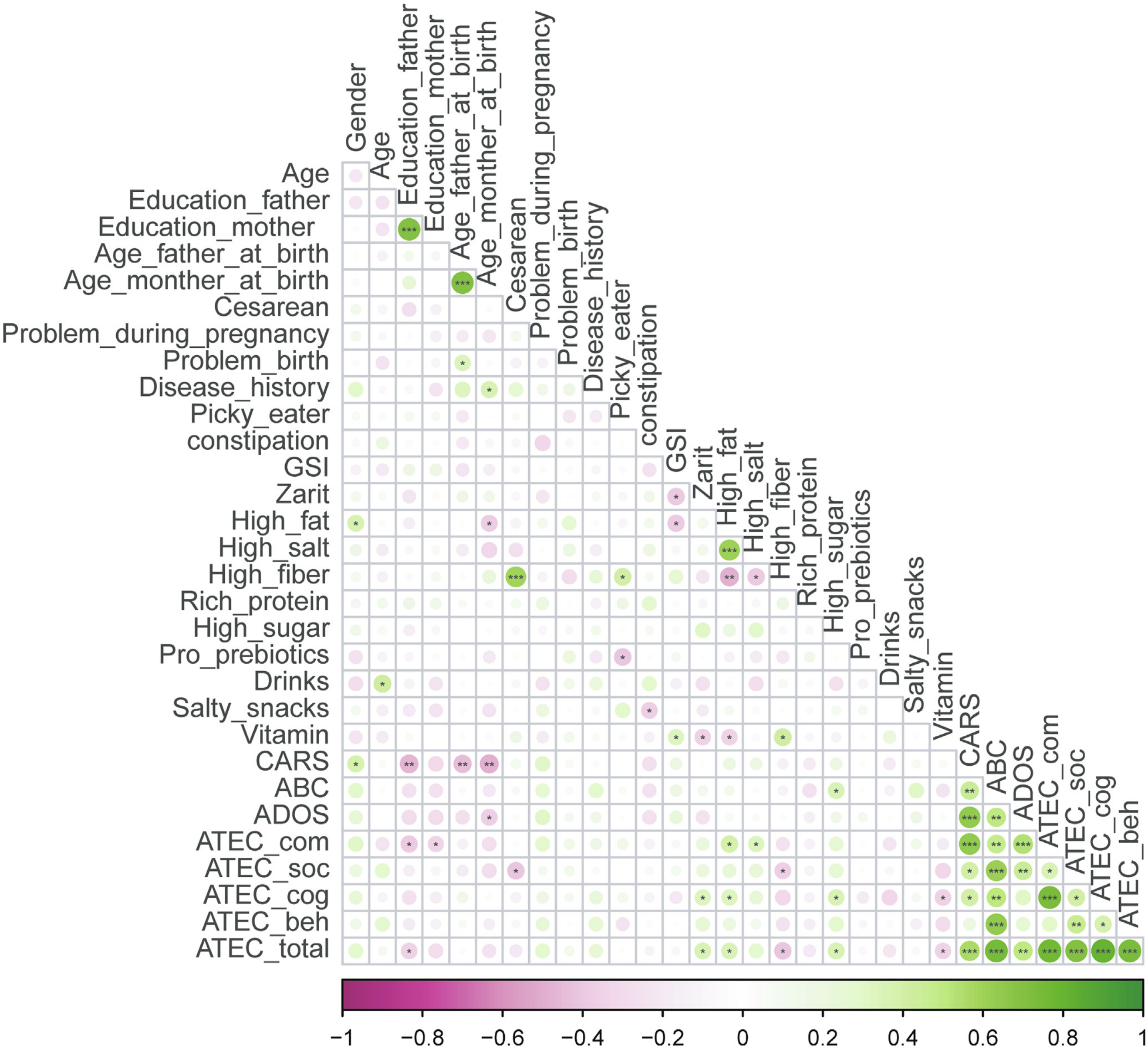



**Fig. 1.** Spearman's correlation analysis of the association between phenotypic indicators. *$P$ < 0.05, **$P$ < 0.01, and ***$P$ < 0.001. In this figure, red circles represent a negative correlation, and green circles represent a negative correlation. The larger the circle, the more significant the correlation.

genus-level enterotypes, characterized by a higher abundance of *Coprococcus*, *Ruminococcus*, and *Faecalibacterium* in Cluster 1, and the *[Ruminococcus] gnavus* and *[Clostridium] innocuum* group in Cluster 2 (Wilcoxon rank sum test, Supplementary Table 1, $P$ < 0.1). Interestingly, the enterotype was also highly correlated with the sample group (Chi-square test, $P$ = 0.0002), with most TD samples belonging to cluster 1 and most ASD samples belonging to cluster 2 (Fig. 2(**F**)).

To explore the differences in microbial community connections between the ASD and TD groups, we constructed a co-abundance network at the genus level using sparCC (Fig. 3). As shown in this figure, the network correlations between ASD and TD are different. To quantify this difference, we calculated the centrality of the edges (connections) and nodes (genera) of the two microbial networks. The ASD and TD groups had four overlapping edges, 62 and 67 of which were unique to the ASD and TD groups, respectively (Fig. S4). Several harmful bacteria (*Klebsiella*, *Enterococcus*, and *Clostridium*) were grouped to form clusters in ASD, and there were clusters of beneficial bacteria (*Bifidobacterium*, *Roseburia*, and *Coprococcus*) in the TDs. Moreover, compared to ASDs, bacteria in TD samples had a more complex network and more connections with each other.

## Association between clinical phenotypes and intestinal microbiota

First, we assessed whether any of the environmental and clinical factors that differentiated the two groups

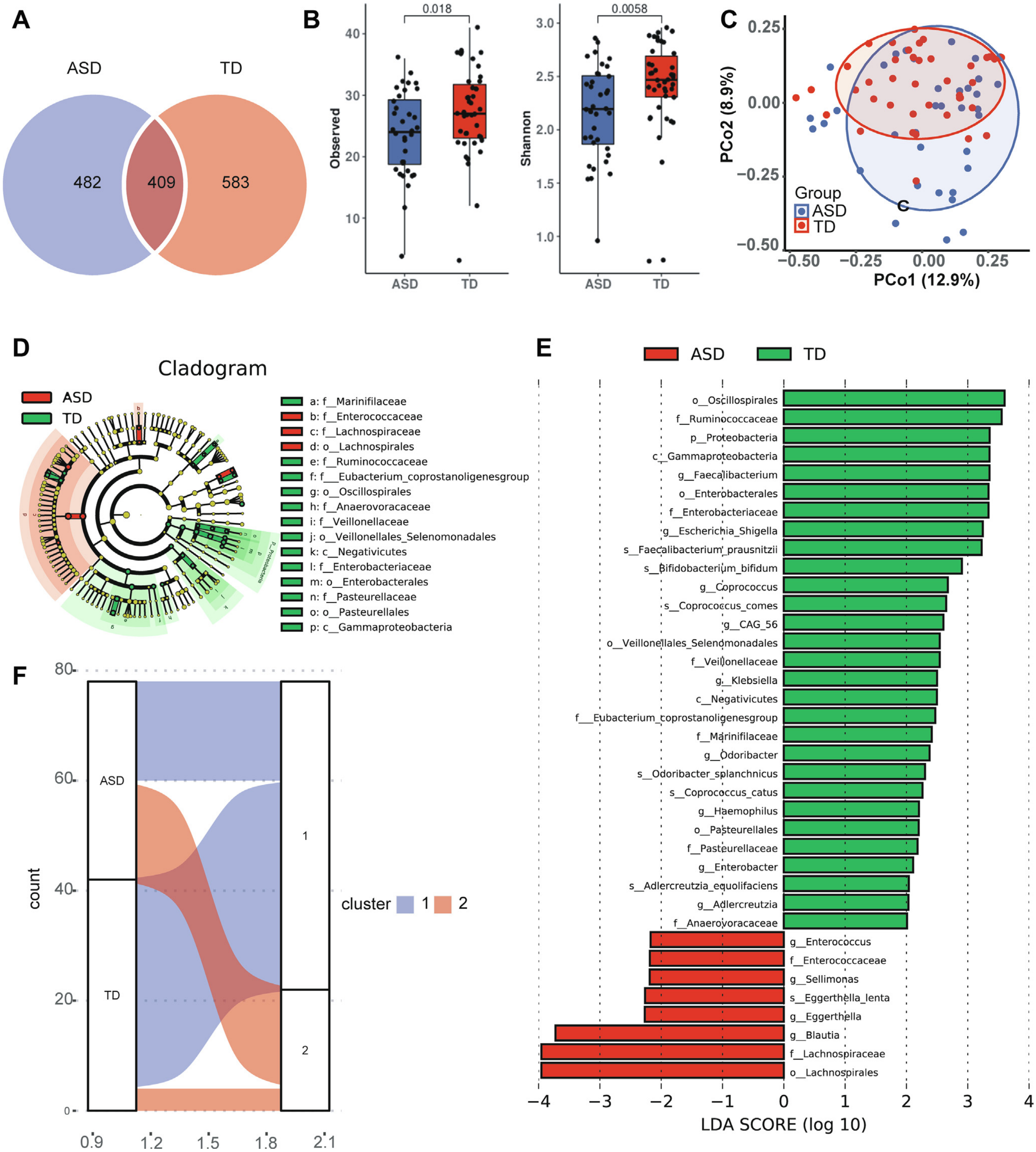
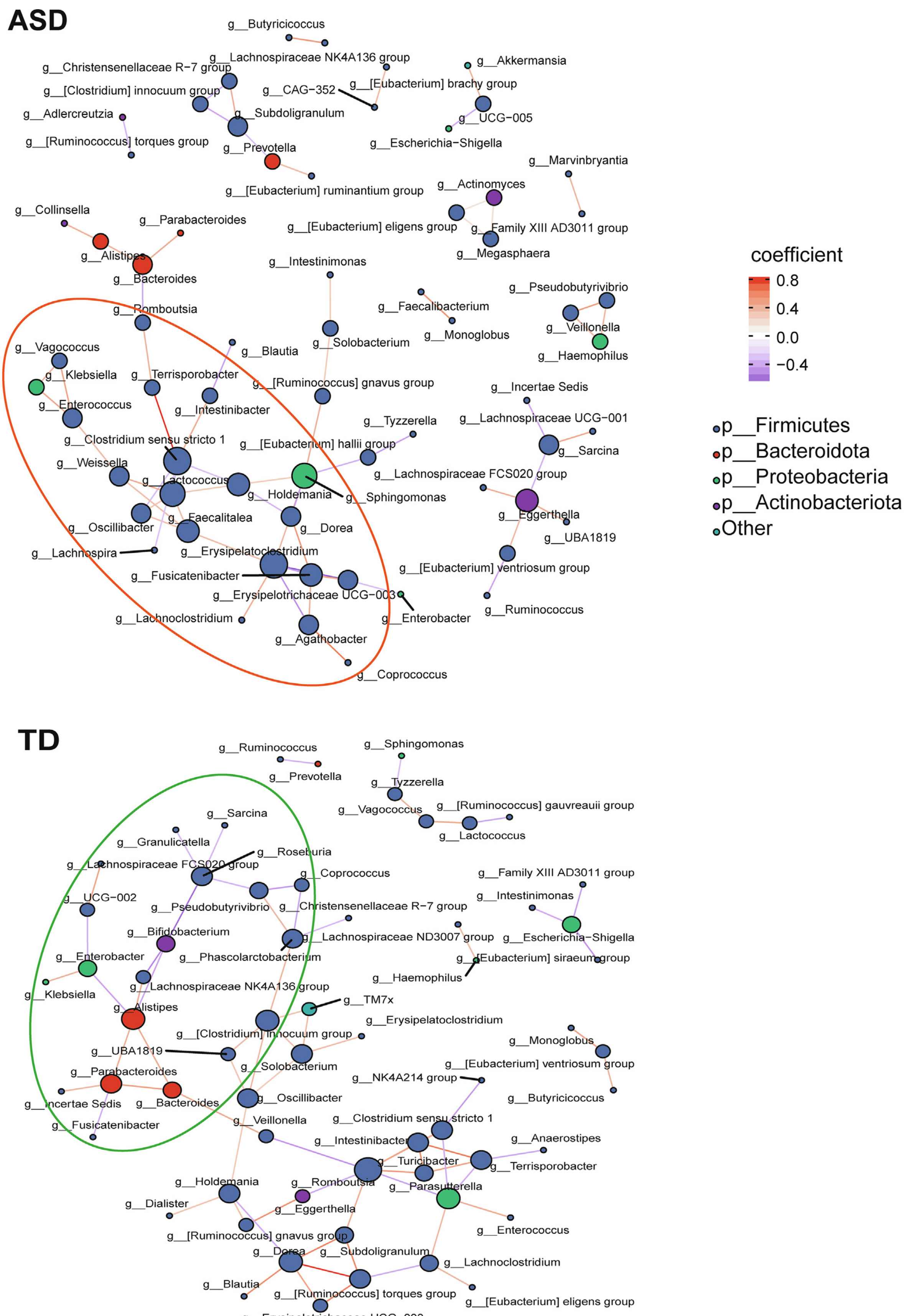



**Fig. 2.** Microbiota changes in children with ASD and GI symptoms based on 16S rRNA data. **(A)** Venn diagram of the observed ASVs in TD and ASD. **(B)** Barplots with standard error showed that children with ASD were characterized by lower microbial diversity compared with TDs, with richness on the left and Shannon index on the right. **(C)** PCoA plot based on Bray–Curtis distance. **(D)** The bacterial taxa that differ between TD and ASD are shown in cladograms produced by LEfSe. **(E)** LDA scores (LDA > 2) for the bacterial taxa diverge in abundance between TD and ASD. **(F)** The interrelationship between the groups and Dirichlet multinomial clusters.

corresponded to the variation in the gut microbiome composition. A canonical correspondence analysis revealed that cesarean section and problems during pregnancy were associated with gut microbiome composition (*P* value = 0.05 and 0.03, respectively). No significant correlation was found between constipation and the microflora composition (Fig. S5).

Spearman's correlation was used to analyze the correlation between the clinical phenotype and species group indicators. Among children with autism, we found


ASD
g__Butyricicoccus
g__Lachnospiraceae NK4A136 group
g__Christensenellaceae R−7 group
g__Akkermansia
g__[Clostridium] innocuum group
g__CAG−352
g__[Eubacterium] brachy group
g__Subdoligranulum
g__Adlercreutzia
g__UCG−005
g__[Ruminococcus] torques group
g__Prevotella
g__Escherichia−Shigella
g__Marvinbryantia
g__[Eubacterium] ruminantium group
g__Actinomyces
g__Collinsella
g__Parabacteroides
g__[Eubacterium] eligens group
g__Family XIII AD3011 group
g__Alistipes
g__Megasphaera
g__Intestinimonas
g__Bacteroides
g__Pseudobutyrivibrio
g__Faecalibacterium
g__Romboutsia
g__Veillonella
g__Monoglobus
g__Solobacterium
g__Vagococcus
g__Blautia
g__Haemophilus
g__Klebsiella
g__Terrisporobacter
g__[Ruminococcus] gnavus group
g__Incertae Sedis
g__Enterococcus
g__Intestinibacter
g__Tyzzerella
g__Lachnospiraceae UCG−001
g__Clostridium sensu stricto 1
g__[Eubacterium] hallii group
g__Sarcina
g__Weissella
g__Lachnospiraceae FCS020 group
g__Lactococcus
g__Holdemania
g__Sphingomonas
g__Eggerthella
g__Faecalitalea
g__Oscillibacter
g__Dorea
g__UBA1819
g__Lachnospira
g__Erysipelatoclostridium
g__[Eubacterium] ventriosum group
g__Fusicatenibacter
g__Erysipelotrichaceae UCG−003
g__Lachnoclostridium
g__Enterobacter
g__Ruminococcus
g__Agathobacter
g__Coprococcus
coefficient
0.8
0.4
0.0
−0.4
p__Firmicutes
p__Bacteroidota
p__Proteobacteria
p__Actinobacteriota
Other
TD
g__Ruminococcus
g__Sphingomonas
g__Prevotella
g__Tyzzerella
g__Vagococcus
g__[Ruminococcus] gauvreauii group
g__Sarcina
g__Lactococcus
g__Granulicatella
g__Roseburia
g__Lachnospiraceae FCS020 group
g__Coprococcus
g__Family XIII AD3011 group
g__UCG−002
g__Intestinimonas
g__Pseudobutyrivibrio
g__Christensenellaceae R−7 group
g__Bifidobacterium
g__Lachnospiraceae ND3007 group
g__Escherichia−Shigella
g__Enterobacter
g__Phascolarctobacterium
g__[Eubacterium] siraeum group
g__Haemophilus
g__Lachnospiraceae NK4A136 group
g__Klebsiella
g__TM7x
g__Alistipes
g__Erysipelatoclostridium
g__[Clostridium] innocuum group
g__Monoglobus
g__UBA1819
g__[Eubacterium] ventriosum group
g__Solobacterium
g__NK4A214 group
g__Parabacteroides
g__Butyricicoccus
g__Oscillibacter
g__Incertae Sedis
g__Bacteroides
g__Veillonella
g__Clostridium sensu stricto 1
g__Fusicatenibacter
g__Anaerostipes
g__Intestinibacter
g__Turicibacter
g__Terrisporobacter
g__Holdemania
g__Romboutsia
g__Parasutterella
g__Dialister
g__Eggerthella
g__Enterococcus
g__[Ruminococcus] gnavus group
g__Dorea
g__Subdoligranulum
g__Lachnoclostridium
g__Blautia
g__[Ruminococcus] torques group
g__[Eubacterium] eligens group
g__Erysipelotrichaceae UCG−003

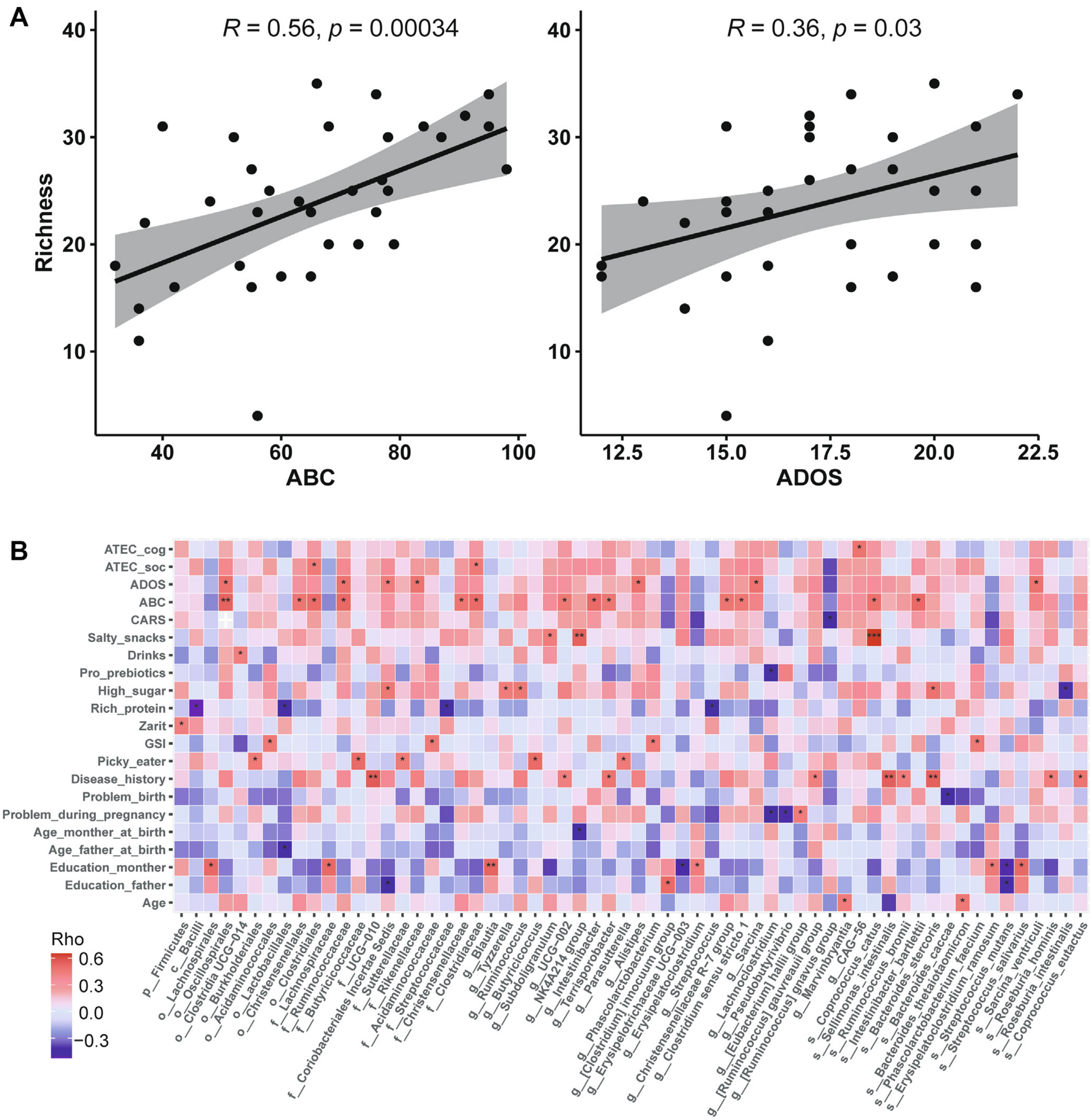



**Fig. 4.** Association between the clinical phenotype and gut microbiome. **(A)** Association between the ABC scores, ADOS scores, and richness based on Spearman's correlation. **(B)** Heatmap shows the Spearman's correlation between phenotypic information and taxa in the ASD group (*$P$ < 0.01, **$P$ < 0.001, and ***$P$ < 0.000).

that the diversity index (richness) was significantly positively correlated with ABC and ADOS scores (Spearman's rho = 0.56, $P$ = 0.34e−03; rho = 0.36, $P$ = 0.03, respectively, Fig. 4(**A**)), and the Shannon index was also significantly positively correlated with the ABC and ADOS scores. Moreover, in the ASD group,

◄

**Fig. 3.** Co-occurrence networks in children with ASD and GI symptoms versus TD children. The size of the node directly relates to the degree of correlation between bacteria and bacteria at the generic level. The color of the connective lines depends on the correlation coefficient, where red lines represent positive correlations and purple lines represent inverse correlations. As shown here, several harmful bacteria were grouped to form clusters in ASD, and there were beneficial bacterial clusters in TDs. These clusters are shown with a red circle on the left and a green circle on the right.

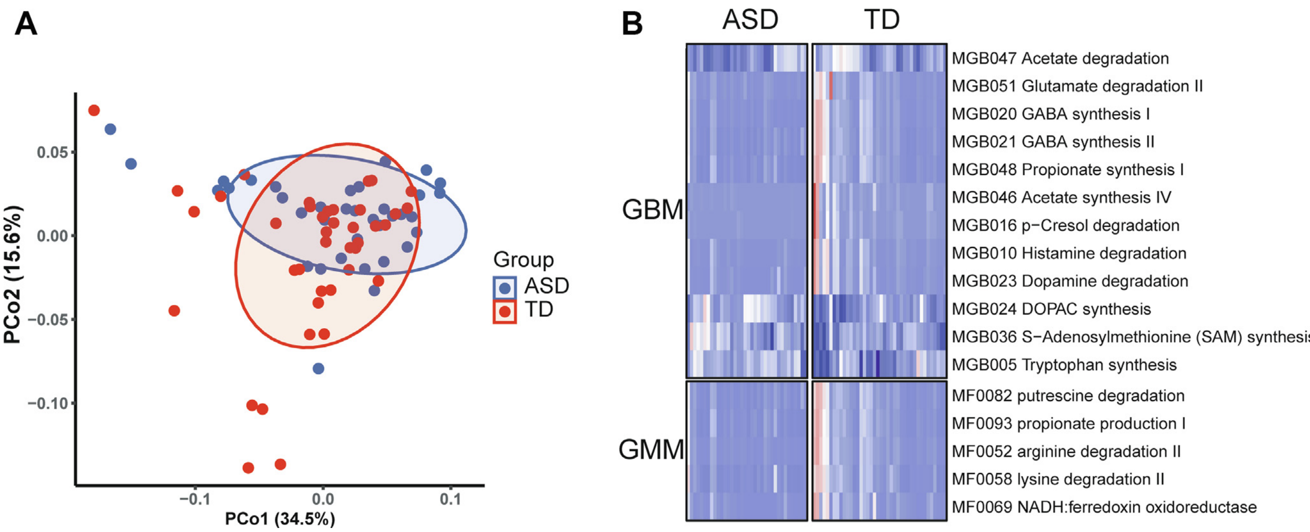



**Fig. 5.** Function prediction using PICRUSt based on KO abundance and discovery. **(A)** PCoA based on the Bray–Curtis distances of KEGG modules in ASD and TD. **(B)** Heatmap shows Gut metabolic module and GBM differences between ASD and TD.

we used Spearman's correlation analysis to associate phenotypes with gut microbes. *P* value < 0.01 and rho absolute value >0.4 was selected as the cutoff. The microbiota of ASDs has some relationship with their clinical phenotypes (Fig. 4(**B**)). Fig. 4(**B**) shows that Clostridiales was positively correlated with the ABC scores (*P* < 0.01). Additionally, salty snacks were positively correlated with *Coprococcus catus* (*P* < 0.000).

## Abnormal microbial functional analysis in ASD with GI symptoms

Permutational multivariate analysis of variance based on KO abundance indicated a significant difference in microbial function between ASD and TD ($R^2$ = 0.032, *P* = 0.024), as illustrated by the PCoA plot (Fig. 5(**A**)).

To further understand the gut functional differences between the two groups, KO abundance was aggregated into the GMM and GBM using omixerRpm. Using FDR < 0.1 as the cutoff, we found that 17 modules were altered in ASD, including 12 GBMs and five GMMs (Fig. 5(**B**)). Of the 12 GBMs, nine were depleted and three were enriched in ASD. ASD depletion modules can be classified into three categories:

- Neurotransmitter-related modules: glutamate degradation, GABA synthesis, dopamine degradation, tryptophan synthesis, and histamine degradation.
- Short-chain fatty acid-related modules: acetate degradation and synthesis as well as propionate production.
- Neurotoxin substance-related modules: lysine degradation, p-cresol degradation, DOPAC synthesis, S-adenosylmethionine synthesis, putrescine degradation, arginine degradation, and NADH: ferredoxin oxidoreductase.

Interestingly, p-cresol was recently reported to be a metabolite that induces autism-like behaviors in mice (Bermudez-Martin et al., 2021). Our results indicate a depletion of p-cresol degradation capacity in ASD.

## Gut microbiota as a biomarker for the prediction of ASD

To evaluate the prediction and classification ability of gut microbiota as biomarkers for ASD, we constructed a classification model based on genus-level abundance data using a Support Vector Machine, which is suitable for small samples and high-dimensional and nonlinear classification problems. Performance of the model was evaluated in another population (12 individuals, including five with ASDs and seven TDs). Before training the model, we used the centered log ratio to transform the abundances at the genus level. Fig. 6(**A**) shows that the prediction model offers a high-resolution ability to predict the ASD status (AUC = 0.88).

To determine the importance of the feature in the data separation task, we used the Shapley Additive exPlanations (SHAP) method based on game theory and the estimation of the Shapley value. SHAP interprets and analyzes a single prediction by calculating the contribution of each feature. A summary diagram of the SHAP values of each feature of each sample (Fig. 6(**B**)) provides an overview of the essential elements of the classifier. The features were sorted according to the sum of the SHAP values and used to display the distribution of the impact of each feature on the output of the model. The taxa that contributed the most to the model were *Bifidobacterium*, *Blautia*, *[Eubacterium] hallii*, *Subdoligranulum*, *Coprococcus*, and *Ruminococcus*, which was in line with the LEfSe results (Fig. 2(**E**)) and differential bacteria in the two enterotypes (Supplementary Table 1).

## DISCUSSION

Our study used well-characterized cohorts to focus on gut microbial changes in patients with ASD and GI symptoms. We identified distinct microbial biomarkers in individuals with ASD and GI symptoms in early childhood. Furthermore, a gut microbial classifier that can

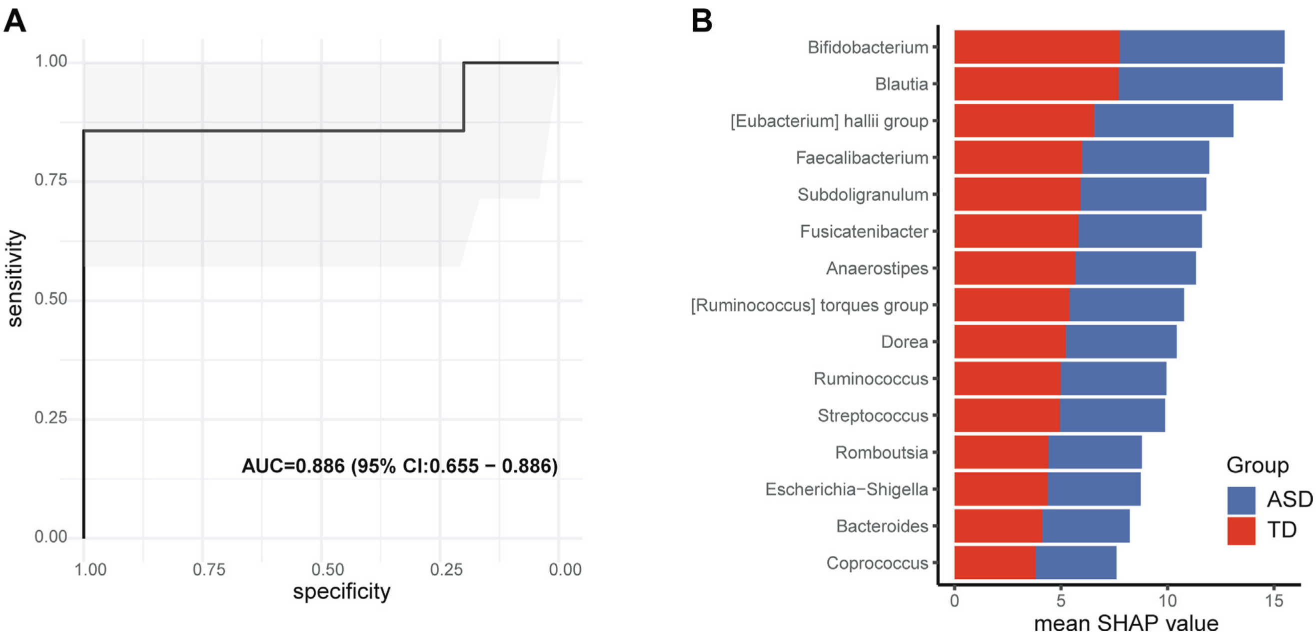



**Fig. 6.** The differential diagnostic potential of microbiological markers for distinguishing ASD and TD are reflected in the validation set AUCs, and differential diagnostic microbial markers for differentiating the two groups are displayed in the figure on the right. **(A)** The ROC curve of TD and ASD classification. ROC curve, receiver operating characteristic curve; AUC, area under the curve. **(B)** Average absolute value of the SHAP values superimposed by ASD and TD for Support Vector Machine for the top 15 features. The length of the red and blue bars represent the enrichment degree of the features in TD and ASD.

accurately distinguish between TD and ASD was identified and validated by our team. Further clinical trials are required to confirm this conclusion. These findings may encourage the development of novel therapies for ASD in addition to their potential for early clinical diagnostic utility.

Growing evidence suggests altered gut microbiota biomarkers in ASD, but we found the microbial characteristics of ASD with GI symptoms of a certain age. Compared to TD, the gut microbiota of ASD patients with GI symptoms exhibited decreased alpha diversity and depletion of butyrate-producing bacteria (e.g., *Faecalibacterium and Coprococcus*), consistent with previous studies (Ding et al., 2020; Iglesias-Vazquez et al., 2020; Zhang et al., 2021). These butyric-producing bacteria have been proven to be essential components of the healthy human microbiota (Miquel et al., 2013; Turroni et al., 2014; Hou et al., 2020). However, some harmful bacteria were enriched in the ASD group, including *Enterococcus*, *Sellimonas*, and *Eggerthella* family. *Eggerthella* has been reported to be associated with depression (Radjabzadeh et al., 2022), and Borkent *et al*. confirmed that there were higher relative abundances of the genus *Eggerthella* in patients with psychiatric disorders (Borkent et al., 2022), which is in line with the findings of our study. Clostridiales, which has been reported to increase significantly in the ASD group (Luna et al., 2017; Berding and Donovan, 2019; Wang et al., 2019; Ding et al., 2020), was positively correlated with ABC scores in our study. Additionally, a co-abundance network analysis revealed a cluster with *Clostridium* as the core of ASD. Thus, the role of these potentially hazardous bacteria in the pathological process of ASD warrants further investigation.

The median male-to-female ratio was 4.2 in autism (Zeidan et al., 2022). One previous study suggests that the prevalence of ASD in boys may be associated with more significant changes in the gut microbiome than in affected girls (Kushak and Winter, 2020). However, there were no differences between boys and girls in our study. One possible reason is the small sample size of autism in our study. According to Wan *et al*., altered bacterial networks are linked to ASD and are characterized by a more complex network with more connections between bacterial species (Wan et al., 2021). However, our study found that the gut microbiota of children with ASD displayed a more basic ecological network, with more clusters formed by potentially hazardous bacteria. A more sophisticated network in TD children can provide a better support system to maintain the stability and balance of the gut microbes.

Many studies have demonstrated that the intestinal community composition of healthy adults does not change significantly over time (Caporaso et al., 2011; Wu et al., 2011), suggesting the overall stability of the ecosystem and enterotype. It is possible to identify or stratify diseases based on gut microbes in the future. Our study found that most TD samples belonged to cluster 1, and most ASD samples belonged to cluster 2. Individuals with ASD in cluster 1 with the same enterotype as most TDs may have a better prognosis, which is worthy of further longitudinal studies. Moreover, cluster 1 was characterized by a higher abundance of *Coprococcus, Ruminococcus*, and *Faecalibacterium*. Long-term perturbation has been demonstrated to profoundly affect intestinal flora, which may lead to enterotype transformation (Ley et al., 2006). Thus, enterotype classification can guide treatment and help understand different therapeutic responses.

Moreover, we found that several GMMs and GBMs were generally decreased in patients with ASD and GI symptoms, including SCFA synthesis/degradation and neurotoxin-related p-cresol degradation. Previous studies have reported that the glutamate system, GABA receptor-mediated function, abnormal dopamine signaling, and tryptophan (a precursor of serotonin synthesis) are particularly relevant to the gut ecosystem in ASD (Paval, 2017; Israelyan and Margolis, 2018; Masuda et al., 2019; Siegel-Ramsay et al., 2021), and our results showed that these modules are lacking in individuals with ASD. Lysine is a ketogenic amino acid that participates in the GBM by synthesizing glutamate as a by-product of its breakdown. We discovered that the lysine degradation module was lower in ASD than in TD, whereas Steven *et al*. observed overexpression of microbial RNAs associated with lysine degradation (Hicks et al., 2018). Microorganisms involved in fermentation produce propionate in the intestine, which helps maintain human health (Reichardt et al., 2014). However, we observed that the propionate production module was lacking in the ASD group. The above findings indicate that abnormal GBMs and GMMs may play an essential role in the pathology of ASD, and this is worth further exploration. Consistent with the results of an animal study, many individuals with ASD lacked the p-cresol degradation module in our study. Core behavioral symptoms of ASD can be selectively induced in mice using the microbial metabolite p-cresol, according to a study by Martin *et al*. The social behavior defect caused by p-cresol depends on alterations in the composition of the microbial community (Bermudez-Martin et al., 2021). Fecal microbial transplantation from ASD patients with GI symptoms to rodents may cause ASD-like behaviors in rodents, and further study can verify this hypothesis. Moreover, it may be possible to treat patients with ASD with intestinal flora that can regulate p-cresol production in the future.

Early diagnosis is critical to the prognosis of ASD, and 3–6 years old is the crucial period for clinical intervention in children with ASD. Our classification model identified *Bifidobacterium*, *Blautia*, *[Eubacterium] hallii*, *Subdoligranulum*, *Coprococcus*, and *Ruminococcus* as robust biomarkers of ASD in 3–6-year-old children with GI symptoms, and the bacteria in this model were consistent with the LEfSe results and differential bacteria in the two enterotypes. Notably, the prediction model was validated in another 12 individuals and showed good performance (AUC = 0.88). To identify the microbial biomarkers most closely related to the pathology of ASD and because recent studies have indicated that GI symptoms and aging may affect gut microbial composition (Rose et al., 2018; Dan et al., 2020; Ronan et al., 2021; Deng et al., 2022), we only chose well-matched children in the discovery and validation sets. A rigorous group entry strategy is valuable for thoroughly assessing the diagnostic effectiveness of microbial markers. In our study, the microbial marker panel produced from the discovery set can accurately differentiate samples from the validation set, indicating that this microbiota-based diagnostic tool may be applied in various clinical settings.

In summary, we described and identified the unique intestinal microbial composition of children with ASD aged 3–6 years with GI symptoms. Moreover, we developed and validated a gut microbial classifier that can effectively distinguish children with ASD aged 3–6 years with GI symptoms from TD children. Since the age of 3–6 years is a crucial stage of ASD diagnosis and intervention, our research results lay a foundation for further development of a clinical diagnosis method based on gut microbes and guide targeted gut microbial intervention.

### Limitations

First, the sample size in this study was small and needs to be further verified with a larger sample in the future. Second, our results do not show a causal relationship between the identified differences in gut microbiota composition and ASD, an inherent limitation of cross-sectional studies. Future longitudinal studies are needed to assess whether microbiological changes lead to improvements in the clinical symptoms. In addition, due to limited research funding, we did not conduct metabolomics and neurotransmitter testing in this study, which limited the interpretation of functional module changes in the ASD group. It is recommended to apply multiple omics methods in future research to gain a clearer insight into the pathological changes in ASDs with gastrointestinal symptoms.

## AUTHOR CONTRIBUTIONS

Yi Zheng and Fan He designed the study. Yingxin Zhao conducted a systematic literature search, analyzed, and wrote this manuscript. Fanchao Meng, Xu Chen, Tianyi Chang, and Huanhuan Huang helped recruit children. Fanchao Meng conducted the ADOS assessment for all subjects. Yaping Wang provided consultation on gut microbiome research and reviewed the manuscript. Yingxin Zhao and Tianyi Chang collected CRF information and stool samples and entered the data. Fan He and Yi Zheng critically reviewed the manuscript for intellectual content, approved the final version, and had access to data. All authors contributed to the article and approved the submitted version.

## ETHICS APPROVAL

Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin. This study is approved by the Ethics Committee of Beijing Anding Hospital, affiliated with Capital Medical University, with ethical approval number 2021 (61). The study respects the principles established in the Helsinki Declaration. Written consent from all the children's parents or legal guardians was obtained.

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## POTENTIAL CONFLICTS OF INTEREST

According to the authors, we carried out this study without any financial or commercial ties that might be construed as a potential conflict of interest.

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## APPENDIX A. SUPPLEMENTARY DATA

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