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Original Research

Characteristics of the Gut Microbiota in Young Adults with Autism Spectrum Disorder

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Abstract

Background: Although the characteristics of the gut microbiota of children with autism spectrum disorder (ASD) have been well studied, those of young adults with ASD have seldom been reported. **Methods**: Using 16S rRNA gene sequencing, we characterized the gut microbiota of 19 young adults with ASD and compared them with that of 19 healthy adults. A random forest prediction model was used to distinguish between the two groups at the genus level. **Results**: The abundance levels of one phylum, seven families, and 18 genera in adults with ASD were significantly different from those of controls. The genus *Phascolarctobacterium* was significantly enriched in adults with ASD, which might elicit ASD-like behavior through production of propionate. In addition, a random forest model identified 15 genera that could distinguish adults with ASD from healthy controls with areas under the receiver operating curve of 92.86%, and ten of them were biomarkers identified by LEfSe. **Conclusions**: Our results identified specific gut bacteria associated with ASD, and the successful application of certain genera in the prediction model further supports the association between gut microbiota and ASD.

Keywords: autism spectrum disorder; gut microbiota; *Phascolarctobacterium*; random forest analysis

1. Introduction

Autism spectrum disorder (ASD) is clinically manifested as impairments in language development and social interaction, as well as behavioral stereotypes and narrow interests [1]. The onset of ASD generally begins at infancy and persists into adolescence and adulthood, with a worldwide prevalence in children of approximately 6.25 per 1000. Additionally, the incidence of ASD varies from country to country. For instance, it is about 18.5 per 1000 in the United States [2], and approximately 2.9 per 1000 in China [3].

To date, the exact etiology and pathogenesis of ASD remain unclear [4,5], but this condition is generally regarded as the result of genetic and environmental interaction [6]. Though hundreds of genes have been associated with ASD, they only account for 10–20% of ASD cases [7]. The occurrence of ASD may also be attributed to maternal exposure to toxic chemicals in the environment during pregnancy [8]. Furthermore, delivery and breastfeeding patterns may be associated with ASD [9–11].

Advances in research into gut microbial ecology have linked gut microbiota with the health of the host. Once an ecological imbalance in the gut microbiota occurs, various diseases, including ASD, can develop [12]. In this process, the brain-gut axis plays an important role [9,13–15]. Accordingly, fecal microbiota transplants (FMT) have demonstrated long-term efficacy in improving gastrointestinal (GI) and behavioral symptoms of ASD patients by modifying gut microbiota [16,17]. However, previous studies have also had conflicting conclusions. For example, Strati *et al*. [18] reported that *Bacteroidetes* is significantly less abundant in children with ASD, whereas Zhang *et al*. [19] suggested that the *Bacteroidetes* level in Chinese children with ASD is significantly higher. Luna *et al*. [20] indicated that *Blautia* is less abundant in children with autism, whereas Berding *et al*. [21] reported that levels of this bacterium are significantly higher in children with autism.

The accumulating evidence indicates that the gut microbiota changes with the host's age [22,23] and dietary alterations [24]. Previous studies have focused on the characteristics of the gut microbiota in children with ASD, although the microbiota characteristics in adult patients with ASD have rarely been reported. Also, adults with ASD tend to be more obese than the general adult population, for both young [25] and old subjects [26]. In a previous study, we compared the gut microbiota of adults with ASD to non-ASD obese adults and found that the propionate-producing species *Phascolarctobacterium succinatutens* is enriched in adults with ASD, whereas *Dialister succinatiphilus* is en-

Table 1. The alpha analysis of two groups.

Group	Sample	ASV	Coverage	Richness		Evenness	Diversity
				Chao	ACE	Simpsoneven	Shannon
ASD	19	1210 ± 67.1	0.99996	1214.75 ± 67.4	1215.27 ± 67.3	0.0589 ± 0.015	5.23 ± 0.628
Control	19	923 ± 44.5	0.99999	923.77 ± 44.3	923.96 ± 44.4	0.0842 ± 0.051	5.20 ± 0.623
p-value		0.0058		0.0057	0.0056	0.0156	0.6283

Note: data for ASV, Richness and Evenness were presented as Mean ± SD. ASV, amplicon sequence variant; Chao, A nonparametric estimator of the species richness of a community proposed by Anne Chao; ACE, Abundance-based coverage estimator; ASD, Autism spectrum disorder.

riched in adults with obesity [27]. In our samples, only 2 of 21 adults with ASD were obese, and we did not compare the gut microbiota of autistic and non-autistic normal-weight adults.

In the present study, we compared the gut microbiota of 19 young autistic Chinese adults to those of 19 healthy Chinese adults. All subjects lived in Jinan, Shandong Province, and the dietary habits of this region are characterized by high intake of meat and seafood, with both wheat and rice as staple foods [28,29].

2. Materials and Methods

2.1 Collection of Stool Samples

Nineteen patients diagnosed with ASD and 19 healthy adults were recruited from Jinan, Shandong Province, China. The patients with ASD were diagnosed in childhood by clinicians according to the diagnostic criteria for childhood autism in the International Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) (World Health Organization, 1993). Patients with schizophrenia or other psychoses were excluded. The gender-matched, healthy controls that did not suffer from ASD, other neurodevelopmental disorders, or neuropsychiatric disease, were recruited from community residents of Jinan. All the subjects were of Han nationality, and had not taken antibiotics or probiotics for one month prior to fecal-sample collection.

Stool specimens were collected into fecal sample preservation solution of the MicroLockerT stool sample collector (Jiangsu YIMI Biotech Inc., Taizhou, China) and transferred to the laboratory within three hours at room temperature.

2.2 16S rRNA Gene Sequencing

The genomic DNA of the stool samples was extracted with the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The V3–V4 region of the 16S rRNA gene was amplified using primers 338F and 806R [30], then purified using an AxyPrep DNA Gel Extraction kit (AXYGEN, Union City, CA, USA), and pooled. The 2 × 300 paired-end sequencing was performed on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA).

2.3 Bioinformatics and Statistical Analysis

Raw FASTQ files were first imported into QIIME2 (version 2022.2, https://qiime2.org/) [31]. The amplicon sequence variants (ASVs) were constructed by Dada2 plugin of QIIME2 with 100% sequence identity [32]. The alpha diversity was analyzed using the mothur software package (version 1.44.3, https://mothur.org/) [33]. The differences in taxonomic abundance and microbiota functions between the ASD and control groups were analyzed using LEfSe (http://huttenhower.sph.harvard.edu/galaxy/) [34], then *p*-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure (significant as FDR <0.05). The principal coordinate analysis (PCoA), based on the Bray-Curtis distance matrix, and permutational multivariate analysis of variance (PerMANOVA) were used to analyze the differences in gut microbiota between the two groups.

The random forest (RF) model was used to identify specific microbial taxa that distinguished ASDs from controls using the "randomForest" package in R 4.2.2. Seventy percent of samples were randomly selected to train the model, whereas the remaining 30% were used to verify the model's performance. "MeanDecreaseAccuracy" and "MeanDecreaseGini" indicators were used to evaluate the relative importance of each genus in the prediction model. The rfcv function implemented in the "RF" package was applied more than five times for ten-fold cross-validation in order to obtain the minimum number of top-ranking genera for prediction. The predictive power of biomarkers selected from the model was assessed by receiver operating characteristic (ROC) curve analysis followed by calculation of the area under the curve (AUC) using the "pROC" package.

3. Results

3.1 Gut Microbiota Composition in Adult Subjects with and without ASD

Nineteen young adults with ASD (mean age = 21 years, range = 17–32 years, mean body mass index (BMI) = 21.9; sex = 5 females, 14 males) and 19 healthy adults (mean age = 29 years, range = 19–37 years, mean BMI = 24.3; sex = 5 females, 14 males) were involved in the study, with most of the ASD patients having GI symptoms. A total of 1,290,959 (24,290–51,389) sequences from the 38 sam-

Table 2. Major families in ASD and control groups.

Family	ASD	Control	Enriched in	FDR
Acidaminococcaceae	1.83% ± 1.91%*	0.37% ± 0.65%	ASD	0.002006
Akkermansiaceae	1.27% ± 5.33%	0.04% ± 0.13%		
Bacteroidaceae	11.12% ± 13.68%	34.73% ± 15.40%	Control	0.00105
Bifidobacteriaceae	2.63% ± 5.78%	0.5% ± 0.56%		
Enterobacteriaceae	0.69% ± 1.48%	2.56% ± 2.51%	Control	0.002006
Erysipelotrichaceae	3.49% ± 3.00%	0.26% ± 0.27%	ASD	0.000024
Fusobacteriaceae	0.14% ± 0.37%	2.47% ± 7.41%		
Lachnospiraceae	23.24% ± 14.39%	20.53% ± 8.01%		
Peptostreptococcaceae	1.08% ± 1.27%	0.39% ± 0.49%	ASD	0.041808
Prevotellaceae	29.47% ± 29.69%	8.98% ± 13.45%	ASD	0.039061
Rikenellaceae	2.44% ± 8.43%	0.92% ± 0.84%		
Ruminococcaceae	13.37% ± 6.89%	16.16% ± 10.59%		
Selenomonadaceae	0.64% ± 1.03%	3.83% ± 7.10%		
Sutterellaceae	0.79% ± 0.77%	2.28% ± 1.65%	Control	0.005931
Veillonellaceae	1.34% ± 2.38%	0.54% ± 0.76%		

Note: *, data for ASD and Control group were presented as Mean ± SD. ASD, Autism spectrum disorder; FDR, false discovery rate.

ples were obtained. Of these sequences, a total of 24,290 sequences from each sample were analyzed by QIIME2, which identified a total of 1681 ASVs (Table 1).

Approximately 99.88%, 97.71%, and 86.80% of the ASVs were assigned to 11 phyla, 53 families, and 165 genera, respectively. Of the phyla, *Firmicutes* (mean = 46.71%), *Bacteroidetes* (mean = 45.45%), and *Proteobacteria* (mean = 3.86%) were the most prevalent in the microbiota of all subjects. Fifteen families (>1% in at least one group) accounted for 94.05% of all the subjects (Table 2), with *Bacteroidaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Ruminococcaceae* as the four most abundant families (>77.2% of each group). Of the 165 genera, 39 were considered to be major, i.e., >0.5% in at least one group (Table 3), with *Phocaeicola*, *Faecalibacterium*, *Blautia*, and *Anaerobutyricum* present in each of the 38 subjects.

3.2 Bacterial Composition Changes between ASD and Controls

The results of PCoA and PerMANOVA analysis revealed a significant difference in bacterial diversity ($F = 3.642$, $p = 0.0001$) between the ASD and control groups (Fig. 1).

At the phylum level, the *Proteobacteria* were significantly less abundant in the ASD group than in the control group (Fig. 2). At the family level (Table 2), seven major families showed significant differences (48.47% in the ASD group and 49.58% in the control group).

At the genus level (Table 3, Fig. 2), 18 major genera differed significantly between the ASD (55.87%) and control groups (60.64%).

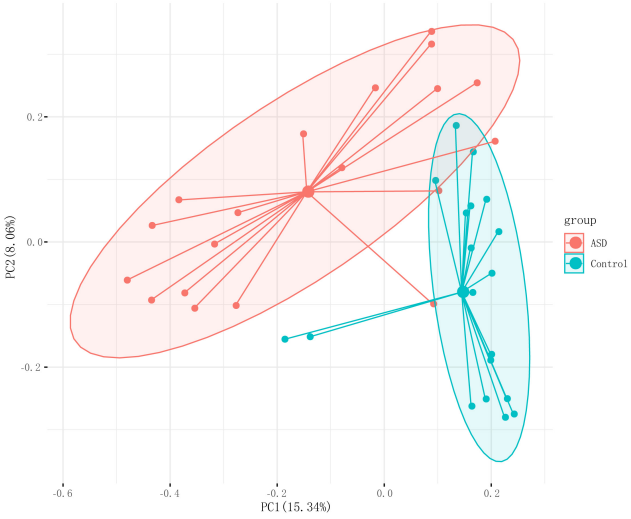

Fig. 1. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity among ASVs of ASD and control groups. Each point represents samples, and the circles surrounding the samples represent 80% confidence interval.

3.3 RF Analysis at the Genus Level

To distinguish the microbiota of young adults with ASD from that of healthy adults, we performed RF analysis with ten-fold cross-validation at the genus level. In general, the optimal model with the lowest error rate had an AUC value of 92.86% (95% confidence interval (CI): 78.86–100%) when 15 genera were included as biomarkers (Fig. 3A,B). Among them (Fig. 3C), ten genera showed a significant difference between the ASD and control groups, including *Phocaeicola*, *Mediterraneibac-*

Table 3. Major genera in ASD and control groups.

Genus	ASD	Control	Enriched in	FDR
Agathobacter	3.93% ± 7.67%*	1.47% ± 1.45%		
Akkermansia	1.27% ± 5.33%	0.04% ± 0.13%		
Alistipes	2.44% ± 8.43%	0.92% ± 0.84%		
Anaerobutyricum	0.22% ± 0.16%	0.8% ± 0.70%	Control	0.007219
Anaerostipes	0.16% ± 0.20%	0.55% ± 0.59%		
Bacteroides	2.69% ± 3.90%	9.13% ± 10.01%	Control	0.008436
Bifidobacterium	2.63% ± 5.78%	0.5% ± 0.56%		
Blautia	3.45% ± 2.82%	2.36% ± 2.83%	ASD	0.043184
Catenibacterium	0.5% ± 1.04%	0.00% ± 0.00%	ASD	0.004553
Clostridium IV	0.71% ± 1.20%	0.14% ± 0.30%		
Clostridium sensu stricto	0.81% ± 1.19%	0.22% ± 0.44%	ASD	0.043184
Clostridium XlVb	0.28% ± 0.50%	0.57% ± 1.29%	Control	0.045836
Coprococcus	0.89% ± 1.81%	0.35% ± 0.71%	ASD	0.049717
Dialister	0.7% ± 1.73%	0.40% ± 0.67%		
Dorea	0.6% ± 0.44%	1.00% ± 2.12%		
Escherichia/Shigella	0.59% ± 1.44%	1.14% ± 1.31%	Control	0.011537
Faecalibacillus	1.37% ± 2.73%	0.17% ± 0.23%	ASD	0.003047
Faecalibacterium	2.87% ± 1.89%	8.62% ± 6.70%	Control	0.019944
Fusicatenibacter	0.55% ± 0.56%	0.41% ± 0.42%		
Fusobacterium	0.14% ± 0.37%	2.47% ± 7.41%		
Gemmiger	1.29% ± 1.63%	0.92% ± 1.06%		
Holdemanella	1.38% ± 2.12%	0.04% ± 0.16%	ASD	0.002805
Klebsiella	0.10% ± 0.42%	1.11% ± 1.95%	Control	0.002826
Lachnospira	3.23% ± 2.96%	3.09% ± 3.31%		
Lachnospiracea_incertae_sedis	1.40% ± 1.78%	0.64% ± 1.04%		
Mediterraneibacter	1.11% ± 1.45%	0.13% ± 0.11%	ASD	0.000748
Megamonas	0.56% ± 1.03%	3.83% ± 7.10%		
Megasphaera	0.61% ± 1.54%	0.03% ± 0.11%		
Parabacteroides	0.67% ± 0.85%	0.94% ± 0.71%		
Parasutterella	0.14% ± 0.29%	1.45% ± 1.89%	Control	0.002254
Phascolarctobacterium	1.83% ± 1.91%	0.37% ± 0.65%	ASD	0.002006
Phocaeicola	8.43% ± 12.72%	25.6% ± 14.86%	Control	0.002006
Prevotella	28.60% ± 29.23%	8.59% ± 13.48%	ASD	0.043184
Prevotellamassilia	0.64% ± 1.00%	0.00% ± 0.00%	ASD	0.007618
Romboutsia	0.62% ± 0.86%	0.36% ± 0.50%		
Roseburia	1.52% ± 1.25%	2.59% ± 2.40%		
Ruminococcus	1.48% ± 1.35%	1.73% ± 1.89%		
Streptococcus	0.61% ± 0.93%	0.43% ± 1.19%		
Sutterella	0.54% ± 0.69%	0.64% ± 1.12%		

Note: *, data for ASD and Control group were presented as Mean ± SD. ASD, Autism spectrum disorder; FDR, false discovery rate.

ter, *Parasutterella*, *Phascolarctobacterium*, *Faecalibacterium*, *Klebsiella*, *Blautia*, *Anaerobutyricum*, *Bacteroides*, and *Holdemanella* (Table 3).

4. Discussion

The gut microbiota needs to be analyzed comprehensively to understand the differences in the bacterial communities in young adults with ASD and those of healthy controls. Our analysis indicated that four genera with an abundance of 14.96% in the ASD subjects and 37.37% in the control subjects comprise the core microbiota (i.e., *Phocaeicola*, *Faecalibacterium*, *Blautia*, and *Anaerobutyricum* are present in each of the 38 subjects). Overall, the microbiota of young adults with ASD differed significantly from that of the healthy controls in one phylum, seven families, and 18 genera.

At the phylum level, the abundance level of *Proteobacteria* was significantly lower in young adults with ASD than in controls (Fig. 2). Strati *et al.* [18] reported that the *Firmicutes/Bacteroidetes* ratio in the feces of individuals with ASD (mean age = 10 years) was significantly higher than that in children without ASD. In contrast, Zhang *et al.* [19] found that the *Firmicutes/Bacteroidetes* ratio

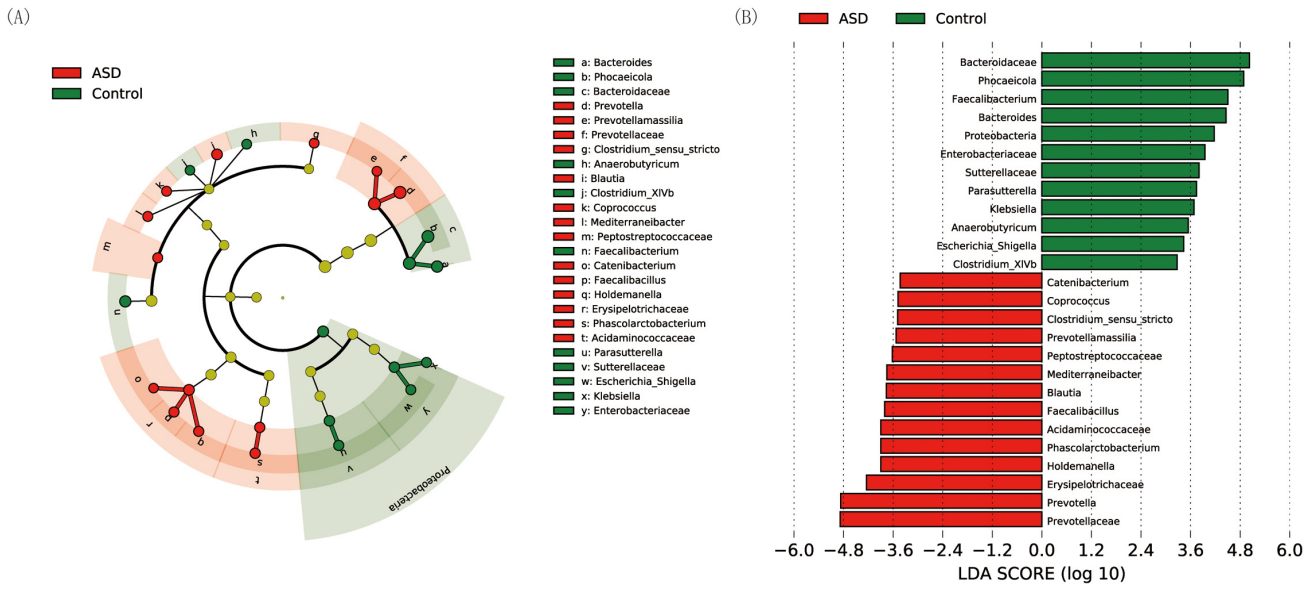

Fig. 2. Comparison of the bacterial taxa in the gut microbiota of ASD and control groups. (A) A cladogram taxonomic representation representing distinct bacterial taxa between the two groups. Red color indicates enrichment in the ASD group, and green indicates enrichment in the control group. (B) A histogram of the linear discriminant analysis (LDA) scores represents significant differences in the abundance of the bacterial taxa between the two groups. Only taxa with significant richness differences (FDR <0.05, computed by LEfSe) between the two groups are shown.

in the feces of 35 Chinese ASD children (mean age = 4.9 years) was significantly lower than that of healthy children. In the present study, the *Firmicutes/Bacteroidetes* ratio in ASD patients (1.10) was slightly higher than that in healthy adults (0.96), suggesting that the difference in ages of the ASD subjects may help explain the conflicting results.

The families *Acidaminococcaceae*, *Erysipelotrichaceae*, *Peptostreptococcacea*e, and *Prevotellaceae* were enriched in ASD subjects, whereas the abundance levels of *Bacteroidacea*e, *Enterobacteriaceae*, and *Sutterellaceae* were significantly lower than those of healthy adults (Table 2). The enrichment of *Acidaminococcaceae* and *Prevotellaceae* and the depletion of *Enterobacteriaceae* were also observed in children with ASD [35], reflecting a common gut microbiota character of ASD, although the depletion of *Bacteroidacea*e showed a contrary trend in children with ASD [35].

The abundance levels of 10 genera (*Blautia*, *Holdemanella*, *Phascolarctobacterium*, etc.) were significantly higher, whereas those of eight genera (*Klebsiella*, *Phocaeicola*, *Parasutterella*, etc.) were significantly lower, in young adults with ASD than in healthy adults (Table 3). Berding *et al*. [21] reported that the levels of *Blautia* are significantly higher in children with ASD (mean age = 4.1 $\pm$ 1.6 years). We detected *Blautia* in all subjects, and its levels were significantly higher (by 1.09%) in the ASD group. *Blautia* has also been reported to be enriched in patients with major depressive disorder [36]. Meanwhile, the other ASD-enriched genus, *Holdemanella*, has been reported to be overrepresented in non-constipated autistic subjects [37]. The relatively higher levels of *Phascolarctobacterium* (1.83% in ASD *vs*. 0.37% in healthy adults, FDR <0.05) and other propionate-producing bacteria in subjects with ASD may be key pathogenic factors associated with ASD symptoms. As is known, *Phascolarctobacterium succinatutens* is able to transform succinate into propionate [38]. Elevated propionate levels contribute to an increased inflammatory profile and disturbed neural connectivity [39]. Because the blood–brain barrier is impaired in individuals with ASD [40], the propionate produced in the intestine may enter the brain and induce autism-like behavior by acting as a neurotoxin [41]. Indeed, propionate has been reported to induce ASD-like behavior [42] or lead to ASD [43].

Ding *et al*. [44] found that children with ASD have significantly lower levels of *Parasutterella*. In the present study, we also observed that the abundance of *Parasutterella* was significantly lower in young adults with ASD than in healthy subjects. A previous study reported that *Parasutterella* produces short-chain fatty acids (SCFA) that are beneficial for improving GI symptoms [45].

Whether the gut microbiome is a cause or consequence of ASD remains controversial [46]. Yap *et al*. [24] reported that the unique gut microbiota character of children with ASD is the result of their dietary preference but not a contributor to ASD. Although the gut microbiota is important in the early stages of neural development [47], the study of Yap *et al*. [24] emphasized the importance of dietary data in ASD-microbiota relationship studies [48]. In the present study, we did not collect dietary data, so the potential ef-

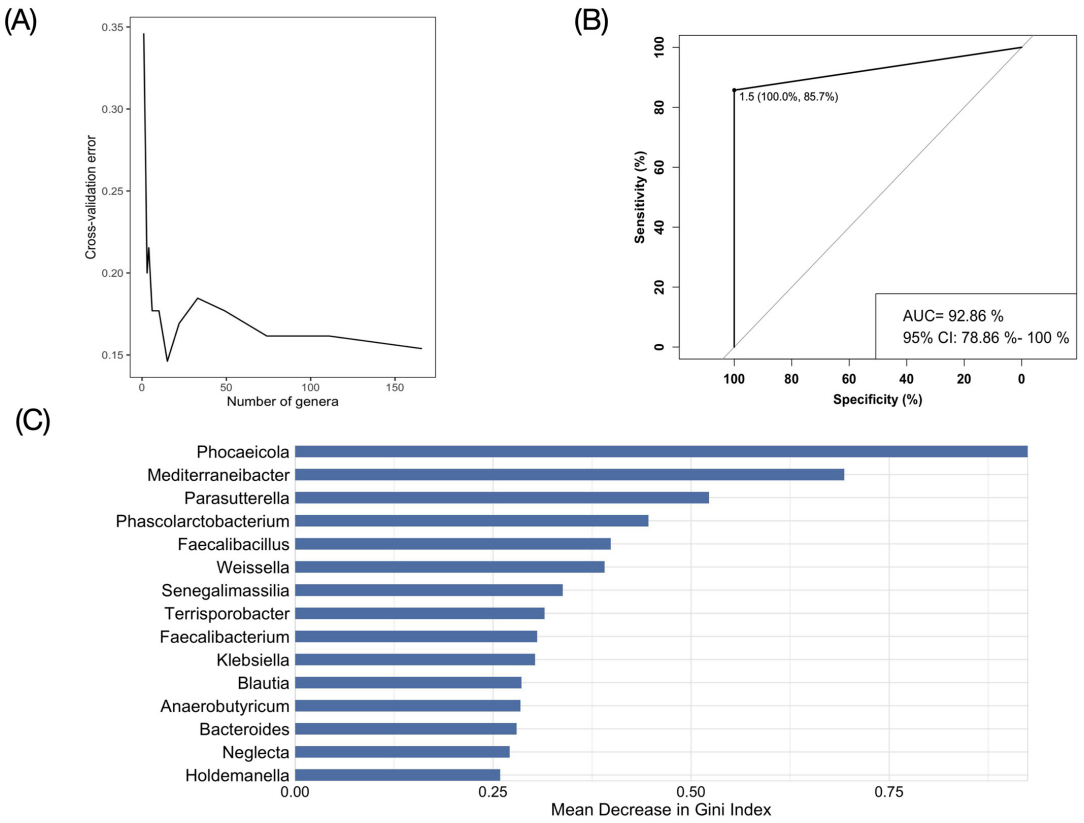

Fig. 3. Random forest model construction and performance analysis. (A) The error rate of random forest model was the lowest when 15 genera were combined through 10-fold cross-validation. (B) The random forest classifier was constructed at the genus level to classify ASDs and controls. (C) The 15 dominant genera were selected as biomarkers according to Gini index of RF model. The length of the bar indicates the importance of the genera. ASD, autism spectrum disorder; AUC, area under receiver operating characteristic curve; 95% CI, 95% confidence interval; RF, random forest; Mean Decrease in Gini Index, the influence of each genus on the heterogeneity of observed values at each node of the classification tree; the greater the value, the greater the importance of the genus.

fect of dietary preferences of the young adults with ASD might have contributed to the observed differences in gut microbiota between the two groups.

To date, the diagnosis of ASD mainly depends on physician observations and responses to questionnaires, such as the Autism Diagnostic Interview-Revised [49]. No biomarkers are currently available. However, because properties of the gut microbiota are associated with ASD, this raises the possibility that ASD can be predicted or diagnosed with the help of gut microbial markers. In the present study, a prediction model for ASD diagnosis using the RF analysis was established. The model identified 15 genera that enable distinguishing between autistic and healthy adults with 100% sensitivity and 85.7% specificity. However, the practical application of using gut microbial properties for the clinical diagnosis of ASD requires the evaluation of more samples and data.

There are some limitations in this study. First, the sample sizes in this study were small, which weakens our statistics and conclusions. Although the changes in the key microbiota revealed in this study were consistent with those of several previous studies, the evaluation of more adults with ASD and healthy controls is needed. Second, dietary data were not collected in our study. Although both the ASD and control groups lived in the same city, dietary differences between groups influence the gut microbiota [24]. Therefore, dietary data should be included in future studies. Third, we did not record intestinal transit time, which is a direct indicator of GI symptoms and plays a role in shaping gut microbiota [50]. Since intestinal transit time can be reflected by the Bristol Stool Scale [51], it would be useful to collect this information to help understand the association between gut microbiota and host health. Finally, the proportion of gram-positive bacteria might have been underestimated since we used a traditional stool DNA extraction method instead of bead-beating to lyse the cell wall of bacteria [52].

5. Conclusions

In summary, we observed that the gut microbiota in young adults with ASD was significantly different from that of healthy adults. Young adults with ASD were associated with more propionate-producing bacteria such as the genus *Phascolarctobacterium* and fewer SCFA-producing bacte-

ria such as the genus *Parasutterella*, than were healthy controls. A prediction model based on RF analysis using 15 genera can distinguish the gut microbiota of autistic adults from that of healthy controls with high sensitivity and specificity, further indicating the association between gut microbiota and ASD.

Availability of Data and Materials

The raw sequence data of gut microbiota have been deposited in the National Omics Data Encyclopedia (NODE, https://www.biosino.org/node/) under accession numbers OEX010410 (adults with ASD) and OEX014604 (healthy adults).

Author Contributions

HZ and QL designed the project. YZ, BC and QZ collected samples. XP, QZ and MG performed experiments. XP, QZ and YW analyzed the data. XP, QZ and HZ drafted the manuscript with other authors involved in revision. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Medical Ethical Committee of the Shanghai Institute of Planned Parenthood Research (NO: PJ2019-17).

Acknowledgment

Not applicable.

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Conflict of Interest

The author Mr. Bin Chen is from the company. He is a volunteer caring for patients with ASD and helps us collecting fecal samples. All the authors, including Bin Chen, had no conflicts of interest.

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