Bioscience of Microbiota, Food and Health Vol. 39 (3), 109–116, 2020

Full Paper

Treatment with the traditional Chinese medicine *BuYang HuanWu Tang* induces alterations that normalize the microbiome in ASD patients

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Received October 7, 2019; Accepted February 14, 2020; Published online in J-STAGE March 6, 2020

Autism spectrum disorders (ASDs) are prevalent neurobiological conditions with complicated causes worldwide. Increasing researcher awareness of ASD and accumulated evidence suggest that the development of ASD may be strongly linked to the dysbiosis of the gut microbiota. In addition, most of the current studies have compared autistic children and neurotypical children or have compared ASD patients before and after antibiotic treatment. Treatment of autism with traditional Chinese medicine (TCM) has increasingly been promoted, but the relationship between its efficacy and intestinal flora has rarely been reported. Under the premise that treatment with the TCM *BuYang HuanWu Tang* is effective, we conducted a comparative bioinformatics analysis to identify the overall changes in gut microbiota in relation to ASD by comparing the intestinal flora before and after treatment with TCM and contrasting the intestinal flora with that of healthy controls. At the phylum level, Proteobacteria showed a significant increase in children with ASD, which may be a signature of dysbiosis in the gut microbiota. At the genus level, *Blautia, Coprococcus* 1, the *Lachnospiraceae* family, and the *Ruminococcaceae* family were found at the lowest levels of relative abundance in children with ASD, whereas the abundances of *Escherichia-Shigella, Klebsiella*, and *Flavonifractor* were significantly increased compared with those in the healthy control group. In sum, this study characterized the alterations of the intestinal microbiome in children with ASD and its normalization after TCM treatment (TCMT), which may provide novel insights into the diagnosis and therapy of ASD.

Key words: autism spectrum disorders, microbiome, traditional Chinese medicine

INTRODUCTION

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders that are highly influenced by strong genetic underpinnings and have their onset in infancy [1]. ASD is a chronic condition that can be lifelong and irreversible [2]. Previous studies suggest that severe social skill deficits and restricted, repetitive, and stereotyped behavior patterns, interests, and activities are core clinical signs. Furthermore, some other features, such as communication deficits, regression, and Asperger Syndrome, contribute to the diagnosis [3]. Although the exact etiology is still unknown, ASDs are universally accepted as the most heritable of neurodevelopmental disorders, with an incidence in males that is significantly higher than that in females [4, 5]. We face difficulties in diagnosis and treatment because of the unknown etiology whereby both genetic and environmental factors play important roles in the pathogenesis of ASD [4].

The microbiota–gut–brain axis is a bidirectional communication pathway between the gastrointestinal (GI) tract and brain, which is important for maintaining a constant internal environment [6–8]. In addition, the gut microbiome is vital to the normal physiologic function of the microbiota–gut–brain axis. Currently, researchers are paying more attention to the relationship between gut microbiota balance and psychological

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2480/)

doi: 10.12938/bmfh.2019-032

diseases. Accumulating evidence suggests that altered gut microbiota and decreased diversity affect both GI function and central nervous system (CNS) physiology by regulating the brain–gut axis [9]. Based on these discoveries, the relationship between depression and dysbiosis of the gut microbiome has been studied, and it highlights the possibility for prevention and therapy [10–13]. Considering the similarity between depression and ASDs, growing studies indicate that there is a strong positive correlation between GI problems and ASD severity, and GI problems are associated with dysbiosis of the gut microbiome [14–16]. A previous study mentioned that gram-negative bacteria, particularly, an increase in the population abundance of *Desulfovibrio and Bacteroides vulgatus*, were observed in autistic children compared with healthy controls [16, 17]. However, gram-positive bacteria, including *Clostridium,* are reported to be present at a higher level in autistic individuals [17–19]. Although several studies have reported that dysbiosis of the gut microbiome has been identified in children with ASDs, the specific bacterial genera or species that are commonly dysregulated have not yet been identified [15]. Therefore, further studies are necessary to confirm specific changes in flora in ASDs, which would then deepen our understanding of the etiology and treatment of ASDs.

To date, most studies have been interested in the genetic, behavioral, and neurological aspects of ASD, and therapies for ASDs have been used clinically with the aim of normalizing patient behaviors instead of addressing the core internal factors [4, 20, 21]. For instance, risperidone treatment is administered to children with ASDs characterized by aggression, temper outbursts, and self-injurious behavior [22]. Behavioral approaches are widely regarded as the most effective methods to address delays and deficits, which are common in ASDs [23]. However, these treatments do not have a substantial therapeutic effect for the core symptoms, and all have some side effects. In traditional Chinese medicine (TCM) theory, it is believed that congenital deficiency (*xiantianbuzu*), deficiency of kidney essence (*shenjingkuixu*), lack of support for the heart (*shenshisuoyang*), dysfunction of unchoking and excretion of the liver (*ganshitiaoda*) are the major facets of autism etiology and pathogenesis. In general, the condition is considered a syndrome called Qi_stagnation, also known as '*Yu*' in TCM. A previous study indicated that an ancient TCM formula can be an effective therapy for depression without any side effects [24]. Therefore, we conducted this study of the TCM *Buyang Huanwu Tang* and observed improvements in clinical symptoms after treatment with this TCM (TCMT). Although there have been studies aimed at analyzing the difference in intestinal florae between children with ASDs and healthy controls, there are few studies comparing conditions before and after treatment [17, 25]. In the present investigation, we collected a total of 48 fecal samples from children with and without ASDs and then pyrosequenced the V3/V4 regions in bacterial 16S rDNA from fecal DNA samples to compare the intestinal flora of children before and after treatment with that of healthy controls. To the best of our knowledge, this study is the first to provide new insights into the application of TCM as a novel therapy for ASDs.

MATERIALS AND METHODS

Patients and samples

The subject population consisted of 14 ASD patients who received TCMT. Additionally, 18 healthy controls were obtained from a children's hospital. This study was approved by the First Affiliated Hospital of Nanchang University (clinical trial number: 2014036). Prior to the study, written informed consent was obtained from parents or guardians, and the study was conducted in accordance with relevant guidelines and regulations.

Subject recruitment

We locally recruited 14 children with ASDs from Jiangxi Provincial Children's Hospital. The enrolled subjects ranged from 1 to 10 years old and did not use any type of antibiotic or antifungal medications for at least 3 months prior to sample collection. The control group included 18 age-matched children who were unrelated to autistic individuals from the hospital. To assess and confirm the ASD symptoms of the children, we asked parents or guardians to fill out the Childhood Autism Rating Scale (CARS). Detailed information on the survey and eligibility criteria can be found in the Supporting Information (Supplemental Table S1).

Sample collection

Fecal samples were collected from the children with ASDs on the day of hospitalization and the day of discharge. The fecal samples for the children with ASDs and healthy controls were collected and stored in germfree containers, and the sample weight was limited to 100–200 g to ensure that there were enough bacteria for culture. All samples were immediately stored in a −80°C environment until DNA extraction. Ultimately, we collected a total of 42 samples from 14 children with ASDs at hospitalization and discharge and 14 healthy controls. Specimens were divided into three groups: Patient or Hospitalized (labeled R, n=14), Traditional Chinese Medicine Affected or Discharged (labeled C, n=14), and Normal or Healthy control (labeled Z, n=14).

DNA extraction PCR amplification

We extracted DNA from fecal samples with a DNeasy Blood & Tissue Kit and followed the protocol recommended by QIAGEN. After genomic DNA extraction was completed, agarose gel electrophoresis (1.0%, w/v) was applied to confirm the efficiency of genomic DNA extraction. We amplified the V3–V4 region of the 16S ribosomal RNA gene, and then specific primers with a barcode were synthesized according to the specified sequencing area. To ensure the veracity and reliability of the subsequent analysis, two conditions needed to be met during the PCR amplification: 1) use of low cyclic amplification whenever possible and 2) use of an same number of cycles for amplification of each sample. The V3–V4 region of the 16S ribosomal RNA gene was amplified using two universal bacterial 16S rRNA gene amplicon PCR primers, 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′), by PCR (95°C for 2 min, followed by 25 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min).

Bioinformatic analysis

Raw paired-end FASTQ files were demultiplexed in QIIME 2 (version 2018.4). Quality control was conducted using DADA2 [26], such as trimming of low-quality regions of sequences, filtering any phiX reads, and removing chimeric sequences. Our 16S rRNA sequences were trimmed at 290 bp for forward read

sequences and 225 bp for reverse read sequences, respectively, based on the Phred score of 33. Four samples with total frequencies of less than 20,000 were excluded from further analysis to avoid potential bias. Representative sequences were aligned with the MAFFT program [27] and used for constructing a phylogenetic tree in FastTree [28]. Diversity metrics were calculated with a sampling depth of 22,560 using the core-metrics-phylogenetic plugin within QIIME 2. The Naive Bayes method was performed to train the SILVA database (version 132) [29], and then taxonomy was assigned to all representative sequences.

Statistical analysis

Multiple indices of alpha diversity (Faith's Phylogenetic Diversity [Faith's PD], Shannon index, observed OTUs, and evenness index) were estimated via Kruskal-Wallis tests, and corresponding curves were plotted on the R platform (https://www.r-project.org/). To investigate differences in community membership, Bray-Curtis dissimilarity was shown using three-dimensional principal coordinate analysis (PCoA) in QIIME 2. Differential abundances of gut microbiota at the phylum and genus levels were detected by a nonparametric negative binomial Wald test in the DESeq2 package [30]. A heatmap for significant OTUs was performed with the gplots package in the R platform.

RESULTS

Subject characteristics

A total of 42 fecal samples were collected from the subjects with autism (n=28) and healthy controls (n=14), whose mean ages (± SD) were 3.49 (± 1.2) and 4.56 (± 1.15) years, respectively. No organic lesions were found by imaging and electroencephalographic examinations. Parents or guardians were asked to complete the CARS to ensure the diagnosis of ASD (Table 1 and Fig. 1A).

Sequencing data of fecal samples

Initially, we obtained a total of 2,927,889 demultiplexed sequences after processing, with an average of 68,090 counts per sample. Chimera sequences and poor-quality sequences (quality score <20) were removed, and we were left with average frequencies of 26,576 sequences per sample and 2,287 sequences per feature to estimate community richness and diversity. We excluded singletons and classified all sequences into 1,856 features at a 97% similarity level, which represented 17 phyla

Table 1. Summary of subject characteristics

Subjects characteristics	Patient	TCM	Healthy control
Total number of participants	14	14	14
Male/Female	12/2	12/2	13/5
Ages (years)	3.49 ± 1.2	3.49 ± 1.2	4.56 ± 1.15
CARS	40.5	34.9	15
Medication duration	62.57d		-

CARS: childhood Autism Rating Scale; TCM: traditional Chinese medicine.

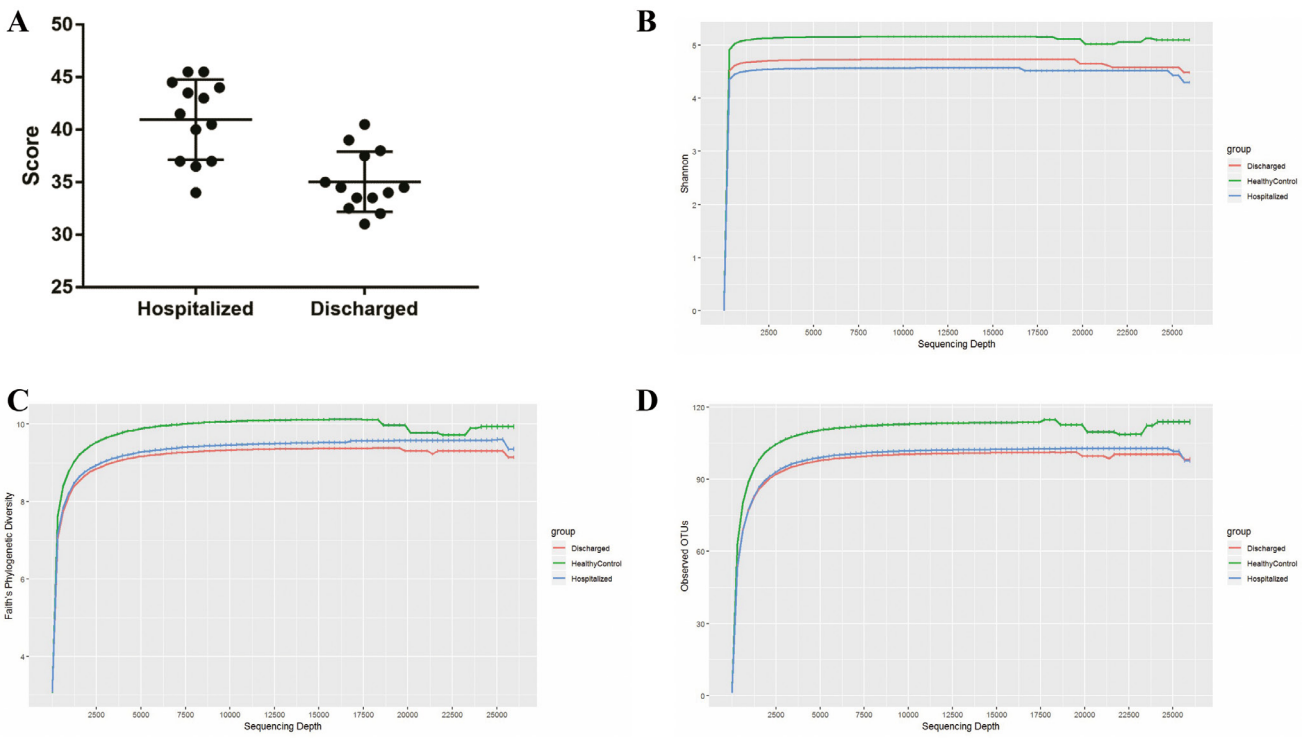

Fig. 1. Alpha diversity of healthy control, hospitalized group, and discharged group. A. The distribution of autism spectrum disorder (ASD) patients' childhood Autism Rating Scale (CARS) scores between hospitalized and discharged patients. B. Shannon index curves for healthy control, hospitalized group, and discharged group. C. Faith's phylogenetic diversity curves for healthy control, hospitalized group, and discharged group. D. Observed operational taxonomic units (OTUs) of healthy control, hospitalized group, and discharged group. Red, green and blue curves represent discharged group, healthy control and hospitalized group, respectively.

doi: 10.12938/bmfh.2019-032

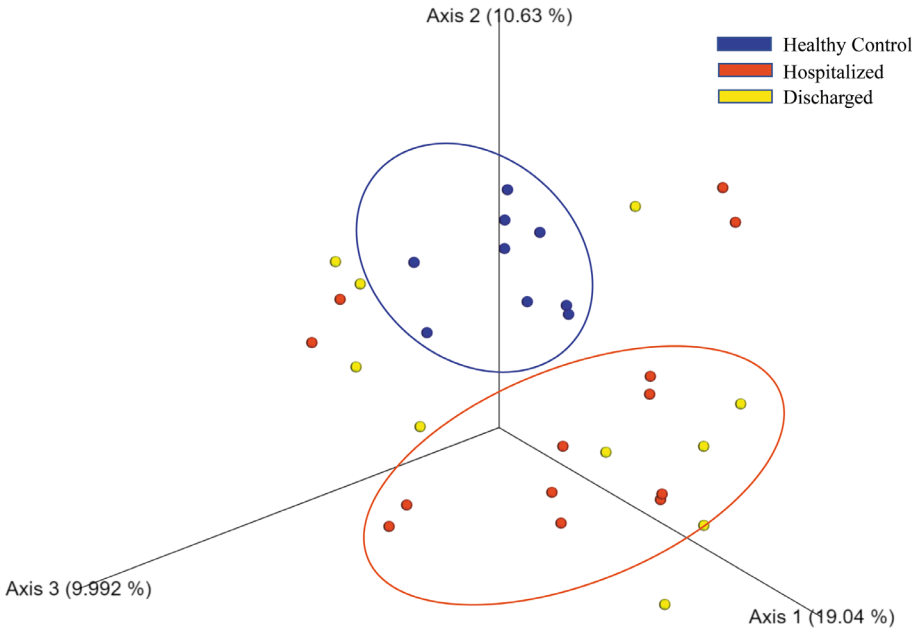

Fig. 2. 3D principal coordinate analysis (PCoA) plot. PCoA plot of the gut microbiota based on the Jaccard and Bray-Curtis dissimilarity. Blue, red, and yellow dots represent Healthy Control, Hospitalized, and Discharged specimens, respectively.

and 265 genera. We took a random sampling of the sequences and then constructed a rarefaction curve by using the number of extracted sequences and the number of OTUs they represented. As the curve flattened out at approximately 4,000 counts, our sequencing of 67,434 counts was deep enough to reflect the microbial information in the samples. Similar to the rarefaction curve, the Shannon-Wiener curve was applied to reflect the bacterial diversity at different sequencing depths.

Autism-associated changes in community diversity and richness

We employed QIIME 2 to compute several alpha and beta diversity metrics to detect community diversity and richness. The metrics computed by default were used in our research. First, the Shannon diversity index showed that the healthy control group had the highest value among the three groups, while the TCMT group had a relatively lower value, Furthermore, the curve of the patient group almost coincided with that of the TCMT group (Fig. 1B). However, the curves demonstrated that the relationship between the observed OTUs and sequencing depth showed the opposite result (Fig. 1C, 1D). The TCMT group had a relatively lower number of observed OTUs than the healthy control group and the patient group, and the curves of the observed OTUs of the other two groups almost coincided, which indicates that treatment with *Buyang Huanwu Tang* is effective in some way and can transform the structure of the microbiome in the human GI tract. As a qualitative measure of community richness that incorporates phylogenetic relationships, we employed Faith's PD between the various features. Based on the Simpson index and Shannon-Wiener index, we also applied Pielou's Evenness as a measure of community evenness using a box plot. Similar to the results for the observed OTUs, the results of applying Pielou's Evenness showed that the TCMT group had the lowest evenness among the three groups, and the qualitative value of the healthy control group was slightly higher than that of the patient group, which indicated that the healthy control group had a higher community evenness than the patient group and the TCMT group. In sum, although the analysis was not statistically significant, it showed a tendency for ASD to affect the composition of the gut microbiome and cause dysbiosis of the microbiome in the GI tract, while TCMT was shown to potentially increase community evenness.

In addition, PCoA based on the Jaccard distance, Bray-Curtis distance, unweighted UniFrac distance, and weighted UniFrac distance was performed to identify whether any differences in the organismal structure of the gut microbiome exist. All three groups showed a cloud of overlapping clustering in both unweighted UniFrac distance and weighted UniFrac distance, although most of the samples could cluster together ($p>0.05$). However, the healthy controls and patient group showed nonoverlapping clustering in both Jaccard distance and Bray-Curtis distance (Fig. 2). Permutational multivariate analysis of variance (PERMANOVA) also identified significant differences in PCoA plots between the healthy control group and the patient group ($p<0.05$), indicating that ASDs can show differences in the organismal structure of the gut microbiome. Interestingly, PERMANOVA identified no significant differences in Bray-Curtis distance and Jaccard distance between the healthy control group and the TCMT group. This pairwise PERMANOVA result suggested that TCMT can normalize the organismal structure of the gut microbiome.

Autism-associated changes in the gut microbiome at the phylum level

To explore the taxonomic composition of the samples, we assigned a pretrained Naive Bayes classifier to the sequences. This classifier was trained on the Greengenes 13_8 99% OTUs, with the sequences trimmed to include only 250 bases from the region of the 16S that was sequenced in this analysis. Ultimately, 8 phyla were classified, and *Firmicutes, Actinobacteria*, and

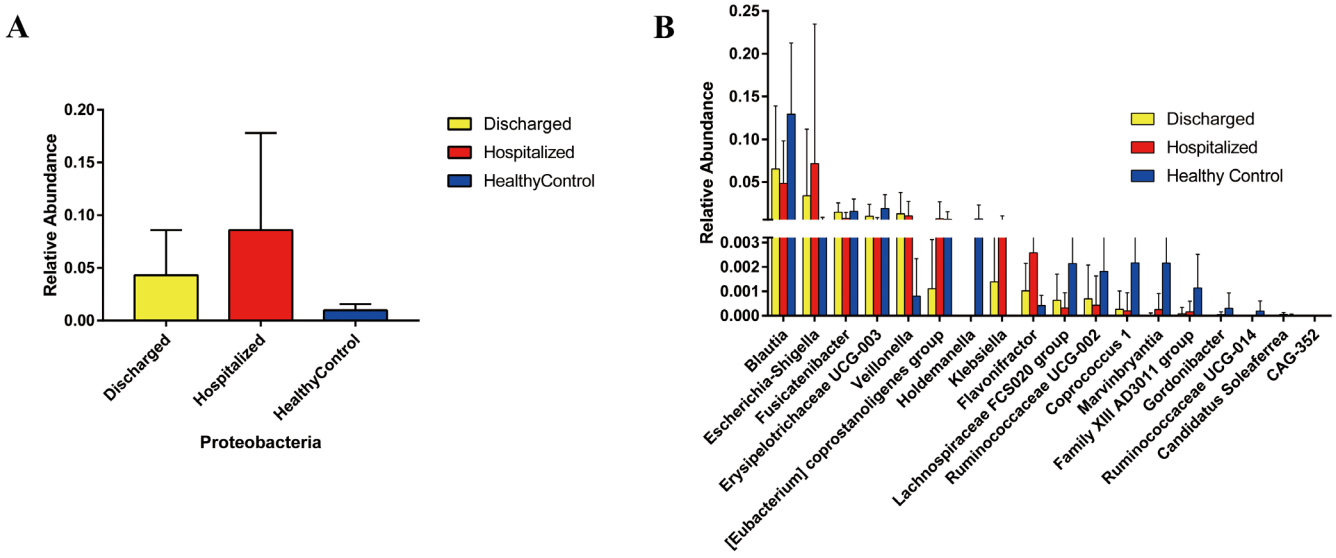

Fig. 3. A. Proteobacteria differing between the Hospitalized, Discharged, and Healthy Control groups. B. Phylum-level bacteria differing between the Hospitalized, Discharged, and Healthy Control groups. The Wilcoxon rank-sum test was applied to compare the abundance at the bacterial phylum and genus levels. Only genera with statistically significant differences are presented ($p<0.05$). Blue, red, and yellow dots represent Healthy Control, Hospitalized, and Discharged specimens, respectively.

Bacteroidetes were the three dominant phyla, as reported in many previous studies [17, 31]. In addition, the phylum *Proteobacteria* was also relatively abundant in autistic children (Fig. 3A). These four phyla comprised an average of 99.3% of the total classifiable sequences across samples. Since this experiment consisted of three groups in total and the abundance data were not normally distributed, the Kruskal-Wallis test was applied to make comparisons of mean abundances between groups. The phylum *Proteobacteria* was found to be more abundant in the patient group than in the healthy control group and the TCMT group, and the difference was statistically significant ($p=0.028$, FDR $Q=0.022$). In addition, the TCMT group had a relatively lower abundance than the patient group but a relatively higher abundance than the healthy control group. The mean abundance of the phylum Bacteroidetes was also determined, and interestingly, that of the TCMT group was the highest. Compared with the healthy control group, the TCMT group had a significantly higher abundance ($p=0.048$); the patient group also had a higher abundance than the healthy control group, but the difference was not statistically significant ($p>0.05$). The phylum *Firmicutes* had the highest abundance, but the Kruskal-Wallis test showed that there was no significant difference in the relative abundance of the phylum *Firmicutes* in any of the three groups.

Autism-associated changes in gut microflora at the genus level

Among 125 genera identified by the pretrained Naive Bayes classifier, the genera *Bifidobacterium*, *Bacteroides*, *Blautia*, and *Faecalibacterium* were the top 4 most abundant genera in all three groups. These 4 genera comprised 51.59%, 48.57%, and 46.35% of the total sequences of the TCMT group, patient group, and healthy control group, respectively (Fig. 3B). Statistically significant differences were found for a total of 18 genera. Eight genera were decreased in the patient group compared with those in the other two groups, including *Blautia*, *Fusicatenibacter*, *Coprococcus* 1, *Lachnospiraceae* FCS020 group, *Ruminococcaceae* UCG-002, *Ruminococcaceae* UCG-014, *Holdemanella*, and *Erysipelotrichacea*e UCG-003 ($p<0.05$). The abundance data showed that the relative abundances of these phyla in the TCMT group were higher than those in the patient group but still lower than those in the healthy control group, with 5 genera showing increases, including *Escherichia-Shigella*, *Klebsiella*, CAG-352, *Candidatus soleaferrea,* and *Flavonifractor* ($p<0.05$). Interestingly, the genus *Escherichia-Shigella* was the fourth most abundant genus in the patient group, representing 7.89% of the total sequences, and was much more abundant in the patient group than in the TCMT group and healthy control group ($p=0.034$). In particular, there was high expression of the genus *Escherichia-Shigella* in one individual, with the genus comprising 56.14% of the total sequences and its high expression revealing a very low community diversity and richness. In addition, the differences in the remaining 6 phyla between the groups were irregular. In contrast to what has been reported, *Veillonella* was more abundant in the patient group than in the healthy control group, while the relative abundance of *Veillonella* was decreased in the patient group compared with that in the TCMT group [32]. In addition, 3 genera, *Gordonibacter*, Family XIII AD3011 group, and *Marvinbryantia* F, showed significant decreases in the patient group compared with the healthy control group, while the relative abundances in the TCMT group were still lower than those in the patient group. *Bifidobacterium* showed a tendency to increase in the TCMT group, but the difference for this genus did not reach statistical significance ($p>0.05$). To show the diversity of bacteria in different individuals more intuitively, we generated a heat map and dendrogram (Fig. 4). There were obvious and impressively strong association between the gut microbiota and the subject grouping. Ten genera, including *Blautia, Fusicatenibacter, Erysipelotrichaceae* UCG-003, *Coprococcus* 1, *Marvinbryantia*, and *Lachnospiraceae* FCS020, were enriched in neurotypical children, while *Klebsiella*, *Veillonella*, and *Flavonifractor* were

doi: 10.12938/bmfh.2019-032

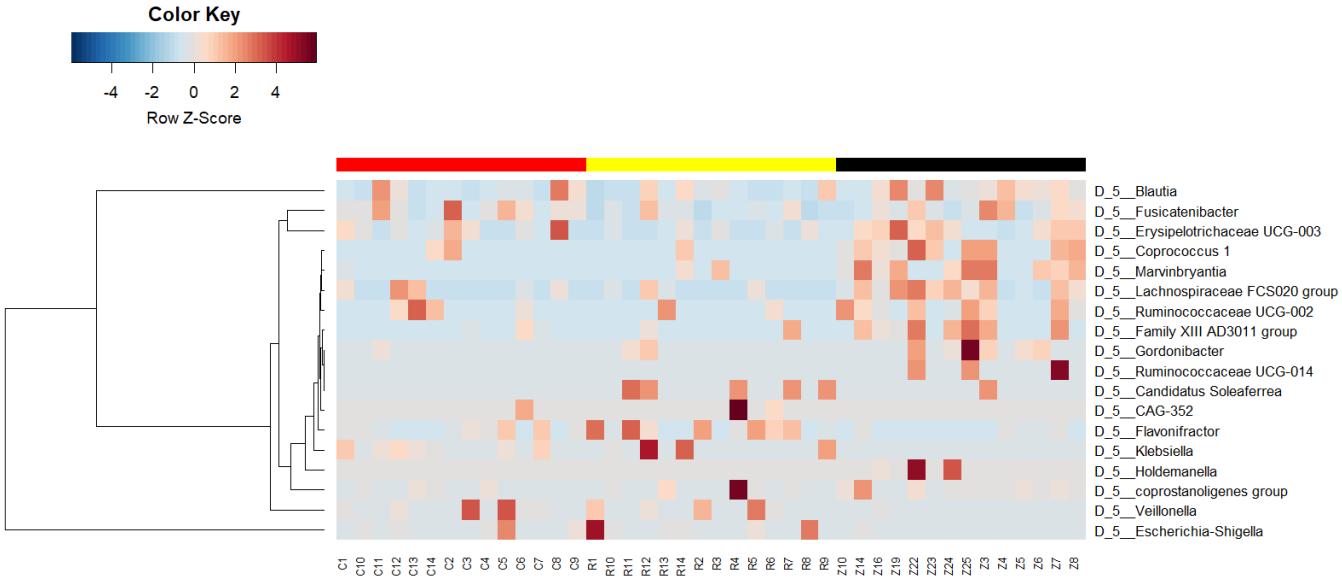

Fig. 4. Heat map profile and dendrogram. Heat map showing positive (red) and negative (blue) associations between each sample (bottom, x-axis) and the relative abundances of bacterial genera (right, y-axis)

exclusively present in children with ASD.

DISCUSSION

In TCM theory, people are divided into nine types of body constitution, among which ASDs are associated with *Qi*_Stagnation, which can lead to "*Yu*" syndrome [33]. As a result of long-term emotional disorder, *Qi*_stagnation forms through personality instability, melancholy, sensitivity, and suspiciousness as the main body state. Among these characteristics, loss of appetite and GI problems are common with this constitution, which shows features that are similar to the typical symptoms of autism. These symptoms not only aggravate the symptoms of autism itself but also lead to other behavioral problems [34]. ASDs can be categorized as the "*Yu*" differentiation grouping of TCM. More precisely, stagnation of liver *Qi* (*Ganqiyujie*) is the main cause of anorexia and GI problems. The clinical manifestations of autistic children are mainly the prominent manifestation of the "liver" losing its regulation of emotion. Therefore, we used the TCM *BuYang HuanWu Tang* to disperse stagnated liver *Qi* to relieve *Qi* stagnation, and the symptoms of the children with ASDs were all improved after treatment, as assessed by the CARS. The main activities of the astragalus membranaceus in the *BuYang HuanWu Tang* decoction are saponins, polysaccharides, and flavonoids. Studies have shown that after oral administration of astragalus membranaceus, its active ingredient, calycosin-7-o-b-d-glucoside (C7G), interacts with intestinal flora and acts as a regulator of intestinal microflora [35].

The gut microbiota plays a crucial role in the regulation of normal gut physiology and immunity. Recently, the signaling between the gut and brain, referred to as the "microbiota–gut–brain axis", has become the focus of research on health and disease. Although there may be some differences in gut microbiome structure between individuals and populations in different regions, there seems to be some balance that is good for health [8]. There is accumulated evidence that dysbiosis in the composition of the gut microbiota can be detected in various neuropsychiatric disorders, such as ASD [36]. Therefore, it is possible to screen for specific bacteria that are associated with autism development to search for a new target for autism treatment and diagnosis. In the present study, we found that the symptoms of children with autism improved to different degrees after TCMT. We subsequently performed a comparative bioinformatics analysis, and several major differences in bacterial commensals were identified.

Alpha and beta diversity analyses showed that the children with ASD tended to have lower bacterial diversity and richness. This fact has been proven by many previous studies [37]. Furthermore, the phylum *Proteobacteria* was found to be statistically significantly more abundant in autistic individuals. Although *Proteobacteria* is the largest phylum of bacteria, it comprises only a small proportion of the bacteria in the GI tracts of healthy individuals. The presence of an increased relative abundance of the phylum *Proteobacteria* is often followed by dysbiosis in the gut microbiota [38]. A total of 18 genera were detected that showed statistically significant differences. In particular, *Escherichia-Shigella* was found to be significantly more abundant in children with ASD, and the propagation of this genus can lead to GI problems. An earlier study examined constipated autistic individuals and characterized them as having a high relative abundance of taxa belonging to the *Escherichia-Shigella* genus [32]. Among the genera detected with significant differences, some match the genera in previous studies. A mouse model of autism showed a reduction in the relative abundances of *Blautia* and *Bifidobacterium*, which are associated with multiple functions of the GI tract as well as bile acid and tryptophan metabolism [39]. Although a decrease in *Blautia* was found in our study, the changes in *Bifidobacterium* were not statistically significant. There was a slight decrease in the relative abundance of *Bifidobacterium* between the patient group and healthy control group, and the value of the TCMT group was higher than that in the patient group, with improvement of symptoms (Supplemental

Table S2). Kang *et al.* found a significantly lower abundance of *Coprococcus* in children with ASD [17]. However, not all of the present results are consistent with those of previous studies. The patient group showed a relatively lower abundance of the *Lachnospiraceae* FCS020 group than the other two groups, while a previous study reported a higher abundance in autistic children with significant changes in fecal and urine metabolites [40]. A recent study reported that *Veillonella* was significantly decreased in children with ASD compared with typically developing children; our study also detected a difference in the relative abundance of *Veillonella* in autistic children, but the results were reversed [41]. As previously mentioned, the change in *Veillonella* in our study is not as typical. Finally, the TCMT group showed an interesting result in the comparisons with the other two groups. While the relative abundances of particular genera in the healthy control group were higher than those in the patient group, the relative abundances in the TCMT group were also higher than those in the patient group but still lower than those in the healthy control group, and vice versa. The number of patients enrolled in this study was insufficient, and this resulted in the observation period being too short to determine efficacy; considering this and the diversity of the intestinal flora, it is difficult to replicate the results. Therefore, these limitations may be responsible for the inconsistency between our results and those of other studies. However, overall, the results of our study, for the most part, showed that the relative abundances of genera in the TCMT group had a tendency to approximate those in the healthy control group, which indicates that our TCMT is effective and can normalize the structure of the microbial community to a certain extent.

CONCLUSION

In conclusion, our study detected the dysbiosis of gut microbiota in children with ASD compared with that in healthy controls and conducted comparisons between before and after the TCMT. The phylum Proteobacteria and a total of 18 genera exhibited statistically significant differences, including *Blautia*, *Fusicatenibacter*, *Escherichia-Shigella*, and *Klebsiella*. Interestingly, *Escherichia-Shigella* was found to be significantly more abundant in children with ASD. In general, the majority of the results of this study matched those of previous similar studies, and there were also some new findings that should prompt further theoretical and empirical investigations and experimental studies in this field.

ACKNOWLEDGMENTS

We thank Jiangxi Provincial Children's Hospital and all staff members who assisted with the study. This study was supported by the National Natural Science Foundation of China (No. 31660035), Key Projects of the Jiangxi Natural Science Foundation (20171ACB20003 and 20192ACBL21042), the Science and Technology Plan Project of the Education Department of Jiangxi Province (GJJ170135), and the Science and Technology Plan Project of the Health and Family Planning Commission of Jiangxi Province (20155115, 20191106, 2018B023 and 2018A093).

REFERENCES

1. Ivanov HY, Stoyanova VK, Popov NT, Vachev TI. 2015. Autism spectrum disorder—a complex genetic disorder. Folia Med (Plovdiv) 57: 19–28. [Medline] [CrossRef]
2. Chaplin AV, Polyakova SI, Kochetkova TO, Sokolova SR, Goltsov AY, Trofimov DY, Efimov BA. 2018. Noncontiguous finished genome sequence of *Megasphaera* sp. ASD88, isolated from faeces of a child with autism spectrum disorder. New Microbes New Infect 22: 13–16. [Medline] [CrossRef]
3. Johnson CP, Myers SM, American Academy of Pediatrics Council on Children with Disabilities. 2007. Identification and evaluation of children with autism spectrum disorders. Pediatrics 120: 1183–1215. [Medline] [CrossRef]
4. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, Miller J, Fedele A, Collins J, Smith K, Lotspeich L, Croen LA, Ozonoff S, Lajonchere C, Grether JK, Risch N. 2011. Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry 68: 1095–1102. [Medline] [CrossRef]
5. Kim YS, Leventhal BL, Koh YJ, Fombonne E, Laska E, Lim EC, Cheon KA, Kim SJ, Kim YK, Lee H, Song DH, Grinker RR. 2011. Prevalence of autism spectrum disorders in a total population sample. Am J Psychiatry 168: 904–912. [Medline] [CrossRef]
6. Mayer EA, Tillisch K, Gupta A. 2015. Gut/brain axis and the microbiota. J Clin Invest 125: 926–938. [Medline] [CrossRef]
7. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. 2015. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 277: 32–48. [Medline] [CrossRef]
8. Cryan JF, O'Mahony SM. 2011. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23: 187–192. [Medline] [CrossRef]
9. Yu M, Jia H, Zhou C, Yang Y, Zhao Y, Yang M, Zou Z. 2017. Variations in gut microbiota and fecal metabolic phenotype associated with depression by 16S rRNA gene sequencing and LC/MS-based metabolomics. J Pharm Biomed Anal 138: 231–239. [Medline] [CrossRef]
10. Foster JA, McVey Neufeld KA. 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36: 305–312. [Medline] [CrossRef]
11. Dinan TG, Cryan JF. 2013. Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol Motil 25: 713–719. [Medline] [CrossRef]
12. Dash S, Clarke G, Berk M, Jacka FN. 2015. The gut microbiome and diet in psychiatry: focus on depression. Curr Opin Psychiatry 28: 1–6. [Medline] [CrossRef]
13. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linløkken A, Wilson R, Rudi K. 2014. Correlation between the human fecal microbiota and depression. Neurogastroenterol Motil 26: 1155–1162. [Medline] [CrossRef]
14. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF, Patterson PH, Mazmanian SK. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155: 1451–1463. [Medline] [CrossRef]
15. Tabouy L, Getselter D, Ziv O, Karpuj M, Tabouy T, Lukic I, Maayouf R, Werbner N, Ben-Amram H, Nuriel-Ohayon M, Koren O, Elliott E. 2018. Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. Brain Behav Immun 73: 310–319. [Medline] [CrossRef]
16. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. 2011. Gastrointestinal flora and gastrointestinal status in children with autism—comparisons to typical children and correlation with autism severity. BMC Gastroenterol 11: 22. [Medline] [CrossRef]
17. Kang DW, Park JG, Ilhan ZE, Wallstrom G, Labaer J, Adams JB, Krajmalnik-Brown R. 2013. Reduced incidence of *Prevotella* and other fermenters in intestinal microflora of autistic children. PLoS One 8: e68322. [Medline] [CrossRef]
18. Song Y, Liu C, Finegold SM. 2004. Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol 70: 6459–6465. [Medline] [CrossRef]
19. Parracho HM, Bingham MO, Gibson GR, McCartney AL. 2005. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. J Med Microbiol 54: 987–991. [Medline] [CrossRef]
20. Kohane IS, McMurry A, Weber G, MacFadden D, Rappaport L, Kunkel L, Bickel J, Wattanasin N, Spence S, Murphy S, Churchill S. 2012. The co-morbidity burden of children and young adults with autism spectrum disorders. PLoS One 7: e33224. [Medline] [CrossRef]
21. Onore C, Careaga M, Ashwood P. 2012. The role of immune dysfunction in the pathophysiology of autism. Brain Behav Immun 26: 383–392. [Medline] [CrossRef]
22. Research Units on Pediatric Psychopharmacology Autism Network. 2005. Risperidone treatment of autistic disorder: longer-term benefits and blinded discontinuation after 6 months. Am J Psychiatry 162: 1361–1369. [Medline] [CrossRef]
23. Sallows GO, Graupner TD. 2005. Intensive behavioral treatment for children with autism: four-year outcome and predictors. Am J Ment Retard 110: 417–438. [Medline] [CrossRef]
24. Wang Y, Fan R, Huang X. 2012. Meta-analysis of the clinical effectiveness of traditional Chinese medicine formula Chaihu-Shugan-San in depression. J Ethnopharmacol 141: 571–577. [Medline] [CrossRef]
25. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, Youn E, Summanen PH, Granpeesheh D, Dixon D, Liu M, Molitoris DR, Green JA 3rd. 2010. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe 16: 444–453. [Medline] [CrossRef]
26. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi

D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72: 5069–5072. [Medline] [CrossRef]
27. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30: 772–780. [Medline] [CrossRef]
28. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26: 1641–1650. [Medline] [CrossRef]
29. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41: D590–D596. [Medline] [CrossRef]
30. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15: 550. [Medline] [CrossRef]
31. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. 2005. Diversity of the human intestinal microbial flora. Science 308: 1635–1638. [Medline] [CrossRef]
32. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, Jousson O, Leoncini S, Renzi D, Calabrò A, De Filippo C. 2017. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome 5: 24. [Medline] [CrossRef]
33. Li M, Mo S, Lv Y, Tang Z, Dong J. 2017. A study of traditional Chinese medicine body constitution associated with overweight, obesity, and underweight. Evid Based Complement Alternat Med 2017: 7361896. [Medline] [CrossRef]
34. Narasingharao K, Pradhan B, Navaneetham J. 2016. Sleep disorder, gastrointestinal problems and behaviour problems seen in autism spectrum disorder children and yoga as therapy: a descriptive review. J Clin Diagn Res 10: VE01–VE03. [Medline]
35. Ruan JQ, Li S, Li YP, Wu WJ, Lee SM, Yan R. 2015. The presystemic interplay between gut microbiota and orally administered calycosin-7-O-β-D-glucoside. Drug Metab Dispos 43: 1601–1611. [Medline] [CrossRef]
36. Sherwin E, Sandhu KV, Dinan TG, Cryan JF. 2016. May the force be with you: the light and dark sides of the microbiota-gut-brain axis in neuropsychiatry. CNS Drugs 30: 1019–1041. [Medline] [CrossRef]
37. Mangiola F, Ianiro G, Franceschi F, Fagiuoli S, Gasbarrini G, Gasbarrini A. 2016. Gut microbiota in autism and mood disorders. World J Gastroenterol 22: 361–368. [Medline] [CrossRef]
38. Shin NR, Whon TW, Bae JW. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 33: 496–503. [Medline] [CrossRef]
39. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, Flynn I, Khochanskiy D, Moya-Pérez A, Peterson V, Rea K, Murphy K, Makarova O, Buravkov S, Hyland NP, Stanton C, Clarke G, Gahan CGM, Dinan TG, Cryan JF. 2017. Microbiota-related changes in bile acid & tryptophan metabolism are associated with gastrointestinal dysfunction in a mouse model of autism. EBioMedicine 24: 166–178. [Medline] [CrossRef]
40. Grimaldi R, Gibson GR, Vulevic J, Giallourou N, Castro-Mejía JL, Hansen LH, Leigh Gibson E, Nielsen DS, Costabile A. 2018. A prebiotic intervention study in children with autism spectrum disorders (ASDs). Microbiome 6: 133. [Medline] [CrossRef]
41. Zhang M, Ma W, Zhang J, He Y, Wang J. 2018. Analysis of gut microbiota profiles and microbe-disease associations in children with autism spectrum disorders in China. Sci Rep 8: 13981. [Medline] [CrossRef]

