Journal of Microbiology (2023) 61:741–753
https://doi.org/10.1007/s12275-023-00069-x

MICROBIAL PHYSIOLOGY AND BIOCHEMISTRY

Fresh Washed Microbiota Transplantation Alters Gut Microbiota Metabolites to Ameliorate Sleeping Disorder Symptom of Autistic Children

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Received: 21 May 2023 / Revised: 25 July 2023 / Accepted: 30 July 2023 / Published online: 4 September 2023

Abstract

Accumulating studies have raised concerns about gut dysbiosis associating autism spectrum disorder (ASD) and its related symptoms. However, the effect of gut microbiota modification on the Chinese ASD population and its underlying mechanism were still elusive. Herein, we enrolled 24 ASD children to perform the first course of fresh washed microbiota transplantation (WMT), 18 patients decided to participate the second course, 13 of which stayed to participate the third course, and there were 8 patients at the fourth course. Then we evaluated the effects of fresh WMT on these patients and their related symptoms. Our results found that the sleeping disorder symptom was positively interrelated to ASD, fresh WMT significantly alleviated ASD and its sleeping disorder and constipation symptoms. In addition, WMT stably and continuously downregulated *Bacteroides/Flavonifractor/Parasutterella* while upregulated *Prevotella*_9 to decrease toxic metabolic production and improve detoxification by regulating glycolysis/myo-inositol/D-glucuronide/D-glucarate degradation, L-1,2-propanediol degradation, fatty acid β-oxidation. Thus, our results suggested that fresh WMT moderated gut microbiome to improve the behavioral and sleeping disorder symptoms of ASD via decrease toxic metabolic production and improve detoxification. Which thus provides a promising gut ecological strategy for ASD children and its related symptoms treatments.

Keywords Autism spectrum disorder · Washed microbiota transplantation · Sleeping disorder · Detoxification

Introduction

ASD presents as a neuro-developmental disorder disease, which is usually an early onset before 3-year-old and characterized by social activities and communication defects, as well as restricted, repetitive, and stereotyped behavior (American Psychiatric Association, DSM-5 Task Force, 2013). Globally, ASD prevalence was one in 132 persons in 2010, but the trend of ASD prevalence between 1990 and 2010 remains unclear. According to estimates from the Autism and Developmental Disabilities Monitoring Network, the prevalence of ASD in the United States was growing, its estimated prevalence was one in 150, one in 69, one in 59, and one in 54 children in 2000–2002, 2012, 2014, and 2016, respectively (Baio et al., 2018; Christensen et al., 2018; Maenner et al., 2020). According to a nationwide multicenter population-based study in China from 2014 to 2016, the observed overall population prevalence of ASD was 0.29% (Zhou et al., 2020). In addition, the prevalence of children with ASD in China is a growing trend due to improved autism diagnosis and increased

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recognition of autism. These observations indicated that it needs an increasing precaution for the Chinese ASD epidemiology.

High risk factors of ASD at least include inheritance, genetic mutation, threatened preterm labour, and environmental factors (Cerminara et al., 2021; Ghosn et al., 2022; Keshtkarjahromi et al., 2021; Kramer et al., 2015). Recently, the interaction among brain, gut, and microbiota had been highlighted in the physiological and pathological process of neuronal system, which exhibit profound effects on neurological development and disorders (Lynch & Pedersen, 2016; Rhee et al., 2009). In line with these observations, the gastrointestinal disorder was showed as a common clinical sign in ASD patients (Cerminara et al., 2021). In fact, previous studies have highlighted the pathogenetic microbiota and its metabolites correlated with ASD (Liu et al., 2021; Panzer & Lynch, 2020; Roussin et al., 2020; Zhang et al., 2020a). Furthermore, several Chinese groups have also indicated the difference in microbiota composition and their metabolites of the ASD patients and health control donor in the Chinese adolescent population (Hua et al., 2020; Zhang et al., 2020a).

Clinically, there is still no well-defined physiological basis for ASD diagnosis, and there is not pharmaceutical drug available for ASD treatment, and the not-pharmaceutical interventions might rise ongoing debate over their merits (Hunter, 2023). Interestingly, accumulating studies have been showing that FMT functions as the most promising therapeutic approach to rebuild microbiota homeostasis in ASD treatment (Roussin et al., 2020; Vendrik et al., 2020; Żebrowska et al., 2021). Dietary, antibiotic treatment, host individuality, and environment have fundamental effects on microbiota homeostasis (Nitschke et al., 2020; Sommer et al., 2017). Therefore, genetic, dietary, lifestyle, and culture differences between Chinese children and western children would make it difficult for Chinese experts to directly draw the experience of western FMT-related application onto the Chinese ASD children treatment (Wang et al., 2019).

In the earlier period, due to fresh stool or manual and crude preparation of microbiota was used for FMT, it's usually a negative perception for the doctor and the patient, and the quality and dose of delivering microbiota were uncontrollable and hard to track, adverse events of fecal microbiota transplantation at least included diarrhoea, abdominal discomfort/pain/cramping, microbiota related infections and even deaths (Marcella et al., 2021; Zhang et al., 2020c). Thus, the safety of FMT should be paid more attention. To our knowledge, the WMT was designed and developed by the professor Faming Zhang team in 2014 (Cui et al., 2015). Recently, Professor Zhang further showed that viruses and pro-inflammatory metabolites could be washed out during washing process, which in turn made WMT to be more safer, precise and more quality-controllable (Zhang et al., 2020c). Otherwise, the clinical effects and the underlying mechanism of WMT on ASD treatment remain largely unknown.

Our previous studies have been showed that microbiota restoration had ameliorated alopecia areata, nonerosive reflux disease, essential tremor, anti-hypertensive effect, and even improved helicobacter pylori eradication (Liu et al., 2020; Xie et al., 2019; Ye et al., 2020; Zheng et al., 2021; Zhong et al., 2021). In the present study, we performed WMT to further investigate its effect on ASD and its related symptoms treatment. We then performed the 16S microbiota sequence to explore the relationship of gut dysbiosis and ASD, even its related symptoms. We also applied bioinformatic analysis to map and speculate on the action mode of differential genera and their metabolite-related pathways.

Materials and Methods

Subject Recruitment

Patients with the diagnosis of ASD were enrolled to Department of Gastroenterology of the First Affiliated Hospital of Guangdong Pharmaceutical University from June 23, 2019, to August 26, 2020. Inclusion criteria for young ASD patients: (1) Age > 2-year-old; (2) Gender unlimited; (3) Confirmed as ASD; (4) Their parents or guardian have signed informed consent colonoscopy and TET; (5) Their parents or guardian cooperated to evaluate the Aberrant Behavior Checklist. Exclusion criteria for young ASD patients: (1) Age ≤ 2-year-old; (2) Respiratory depression, airway obstruction or hypoxia; (3) Heart disease; (4) The other brain disease; (5) Abnormalities in liver/kidney function; (6) Cancers; (7) Biliary tract disease; (8) Abnormal blood pressure; (9) Hematological disease or infection; (10) Antibiotics used within 24 h before WMT; (11) Drug and/or alcohol abuse.

Clinical Protocol of WMT

WMT treatment was described as our previous study (Zheng et al., 2021). All patients received liquid food and Lactulose Oral Solution (Abbott Biologicals B.V) one day before WMT treatment. In the WMT process, the patients were subjected to intravenous anesthesia by injection of Propofol Medium and Long Chain Fat Emulsion (3 mg/kg, Guangdong Jiabo Pharmaceutical Co. Ltd). In brief, Transendoscopic enteral tubing (TET) tube was placed into the caecum via colonoscopy, followed by 3–5 ml paraffin oil injection to facilitate colonoscopy removal. Disposable endoscopic titanium clips were then used to fix the most proximal loop of TET tube onto the intestinal wall, the second/third loop were fixed onto mucosal fold, and the distal TET tube was fixed on hip. 5–10 ml saline was injected to make sure that

the pipeline is clear and smooth, and the patients held the right lateral position for microbiota delivery (Fig. 1A–F) (Zhang et al., 2020b). Besides, the patient still held the right lateral position for 3 h after transplantation. According to consensus of WMT, we only used fresh healthy microbiota for WMT in the present study, and the process from preparation to transplantation of healthy microbiota was restricted to less than 1 h (Fecal Microbiota Transplantation-standardization Study Group, 2020). All patients were suggested to take light food for at least 2 days after WMT. There was not any adverse event reported after WMT treatment or the follow-up period.

Outcome Measurement

The Aberrant Behavior Checklist (ABC) was evaluated by patient-related parents as described in the previous study (Farmer & Aman, 2020; Kang et al., 2017). The Bristol Stool Scale was assessed by the professional physician as previously described (Lewis & Heaton, 1997). The Sleep Disturbance Scale for Children (SDSC) was assessed by patient-related parents as previously described (Bruni et al., 1996). Intestinal barrier function was evaluated by serum diamine oxidase (DAO), D-lactate, and endotoxin. No patient reported any side effects during WMT treatment. The intestinal lesion criterion was following: zero score represented as normal, one score represented epithelial cell damage and intestinal ischemia, but without gastrointestinal dysfunction. A score of 2 was defined as increased and abnormal intestinal permeability. A score of 3 was defined as epithelial cell damage, intestinal ischemia, and increased and abnormal intestinal permeability. A score of 4 was defined as intestinal bacterial translocation.

DNA Extraction and Sequencing

Fecal DNA was extracted by using the QIAamp Fast DNA Stool Mini Kit (Qiagen) as recommended.16S rRNA gene region-specific primers V4 are 5′-GTGCCAGCMGCCGCGGTAA-3′ (forward) and 5′-GGACTACNVGGGTWTCTAAT-3′ (reverse). All PCR reactions were amplified by using the KAPA HiFi HotStart ReadyMix kit (KAPA Biosystems) as recommended. PCR products (400–450 bp) were collected by using 2% agarose gel electrophoresis. DNA library was prepared by using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina) as recommended. Library was checked its quality and then sequenced by the Illumina MiniSeq (Illumina).

Bioinformatic Analyses

Raw paired-end reads were assembled using flash (Magoč & Salzberg, 2011). The operational taxonomic unit (OTU) were analyzed by usearch and pipeline (Edgar, 2010). Briefly, all reads were merged in one file and deleted duplicates (Edgar, 2010). RDP Classifier was used to classify after OTU sequences were aligned on the silva_132_97_16S.

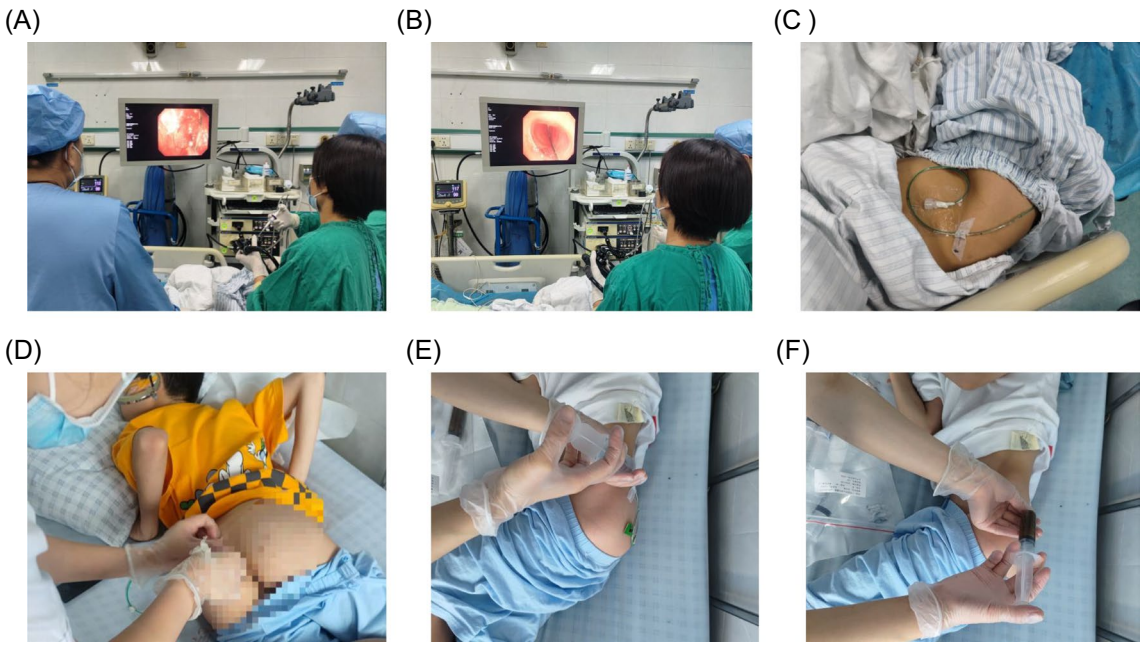


Fig. 1 WMT process. **A** Colonoscopy and Titanium clip fixation. **B** colonoscope withdraw. **C** TET successful insertion. **D** The distal TET tube fixation on hip. **E** Saline delivery. **F** Microbiota delivery

fna database (Cole et al., 2014). FastTree was used to construct Phylogenetic tree (Price et al., 2009).

Functional Profiling Based on Bacterial Taxonomy

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was used to predict relationship between the relevant gene and metagenomics function content from the 16S reads of the rDNA (Douglas et al., 2020). In brief, the qiime R package was used to analyze the 16S reads, 16S.fna database, and the OTUs (Caporaso et al., 2010). OTUs was normalized by normalize_by_copy_number.py, metagenome was predicted by using picrust2_pipeline.py.

Statistical Analyses

The results of alpha/beta diversity, OTUs, Taxa abundance, and LEfSe were visualized and analyzed by using R studio (Version 3.6.3). It was statistical difference when the p value less than 0.05.

Results

Evaluations of WMT Clinical Effect in ASD

In the present study, there were 24 patients underwent the first round WMT, 18 patients in the second round WMT, 13 patients in the third round WMT, and 8 patients in the fourth round WMT. All patients quitted freely during the treatments. We evaluated the ABC score of ASD children between pre-WMT and post-WMT, then found that the ABC score showed a decreased trend in post-WMT (Fig. 2A). We also made a nonparametric analysis of the ABC score between the pre-WMT and post-WMT by Kruskal–Wallis rank sum test, and then found that the ABC score significantly decreased in post-WMT (Fig. 2B). WMT also significantly improved constipation and ameliorated sleeping disorder of ASD patients, and there were statistically significant differences at each intervention node (the ABC score was not statistically significant at the third intervention point). WMT tended to ameliorate intestinal lesion of ASD patients, although there was no statistical difference. WMT significantly up-regulated DAO, D-lactate, and Endotoxin level in ASD patients after the second course of treatment, while other intervention nodes tended to decrease DAO, D-lactate, and Endotoxin level, although there was not statistically difference (Table 1). Moreover, during WMT treatment, the correlation between the ABC score and other clinical indicators were analyzed using Pearson's correlation coefficient. ABC score was significantly and positively correlated with sleeping disorder, while tended to negatively correlate with Bristol/DAO/D-lactate/Endotoxin, and the intestinal condition, although there were not statistically significant (Table 2).

WMT Significantly Modified Gut Microbiota in ASD Patients

The alpha diversity richness of ASD was lower than Donor, and compared with the ASD, the richness of the WMT-1 was significantly increased, although the richness of continuously WMT did not change significantly. Compared with the ASD, the richness of the WMT-2 and WMT-3 increased, while the richness of the WMT-4 decreased, although there was no significant difference (Fig. 3A). Since some participants did not fully participate

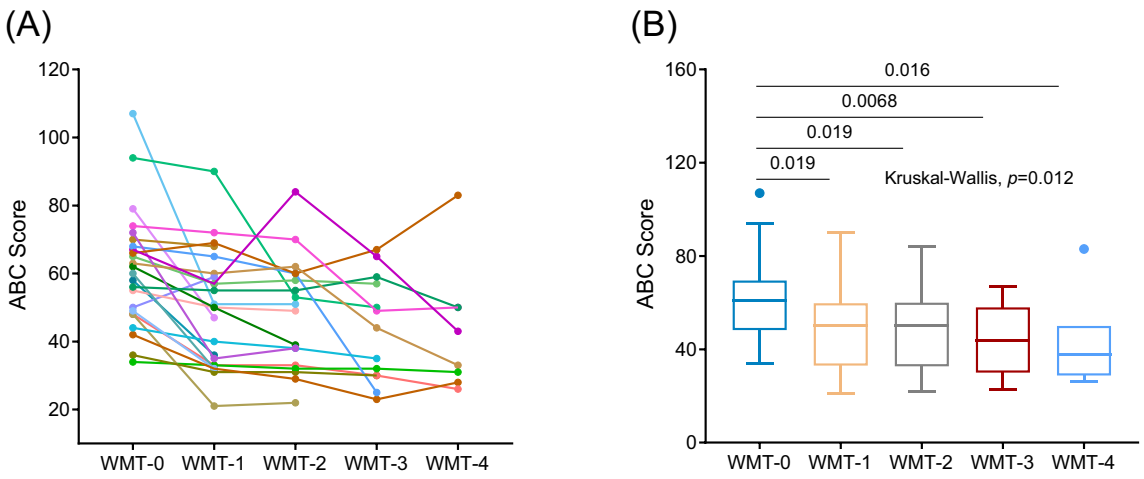


Fig. 2 ABC test evaluates the effect of WMT for ASD treatment. **A** The ABC score was shown as individuals during the WMT treatment. **B** The ABC score was shown as time node during the WMT treatment. n ≧ 8, $*p < 0.05$, $**p < 0.01$

Table 1 The effect of WMT on ASD and its clinical characteristics

Characteristic	Baseline	WMT1	P	Baseline	WMT2	P
ABC	61.13 ± 3.49	48.96 ± 3.45	0.0002	61.17 ± 4.44	48.00 ± 3.85	0.0017
	n = 24	n = 24		n = 18	n = 18	
Sleeping disorder	53.64 ± 2.29	52.23 ± 2.26	0.0167	52.88 ± 2.76	48.13 ± 2.31	0.0007
	n = 22	n = 22		n = 16	n = 16	
Bristol	2.52 ± 0.26	3.05 ± 0.20	0.0146	2.50 ± 0.32	3.56 ± 0.16	0.0050
	n = 21	n = 21		n = 16	n = 16	
DAO	14.18 ± 1.47	14.04 ± 1.04	0.9001	13.474 ± 1.454	20.62 ± 2.27	0.0454
	n = 14	n = 14		n = 11	n = 11	
D-lactate	18.94 ± 1.62	18.81 ± 1.84	0.5302	18.90 ± 1.94	30.09 ± 2.64	0.0039
	n = 14	n = 14		n = 11	n = 11	
Endotoxin	10.58 ± 0.96	10.39 ± 0.85	0.7536	11.06 ± 1.03	16.75 ± 2.23	0.0367
	n = 14	n = 14		n = 11	n = 11	
Intestinal lesion	2.14 ± 0.27	2.29 ± 0.29	0.7551	2.18 ± 0.33	3.09 ± 0.25	0.0335
	n = 14	n = 14		n = 11	n = 11	
Characteristic	Baseline	WMT3	P	Baseline	WMT4	P
ABC	58.23 ± 4.73	43.54 ± 4.27	0.0052	56.25 ± 4.89	43.00 ± 6.62	0.0584
	n = 13	n = 13		n = 8	n = 8	
Sleeping disorder	56.00 ± 2.78	48.92 ± 2.47	0.0016	58.29 ± 3.62	50.00 ± 3.10	0.0360
	n = 13	n = 13		n = 7	n = 7	
Bristol	2.25 ± 0.33	3.58 ± 0.15	0.0077	2.25 ± 0.41	3.69 ± 0.31	0.0350
	n = 12	n = 12		n = 8	n = 8	
DAO	15.12 ± 1.96	15.13 ± 1.25	0.8384	17.04 ± 3.01	16.03 ± 3.05	0.5294
	n = 10	n = 10		n = 6	n = 6	
D-Lactate	18.80 ± 2.30	16.03 ± 2.23	0.9188	21.24 ± 3.16	16.77 ± 2.26	0.2945
	n = 10	n = 10		n = 6	n = 6	
Endotoxin	12.42 ± 1.44	10.87 ± 0.88	0.4755	13.91 ± 2.09	11.38 ± 2.46	0.2945
	n = 10	n = 10		n = 6	n = 6	
Intestinal lesion	2.40 ± 0.34	2.10 ± 0.38	0.5862	2.83 ± 0.40	1.67 ± 0.49	0.3387
	n = 10	n = 10		n = 6	n = 6	

Table 2 Correlation analysis of ABC score and other clinical indicators during WMT treatment

ASD (ABC score)	Sleeping disorder	Bristol	DAO	D-Lactate	Endotoxin	Intestinal lesion
Coefficient						
Pre-WMT	0.14	0.2	−0.18	−0.09	−0.26	0.00
Post-WMT	0.42	−0.09	0.25	0.24	0.23	−0.08
P value						
Pre-WMT	0.54	0.39	0.44	0.71	0.26	1.00
Post-WMT	0.04	0.71	0.30	0.33	0.33	0.76

in all 4 courses of treatment, we analyzed alpha diversity in another 8 patients who fully participated in the 4 courses of treatment, then our results showed that richness of the WMT-4 course increased, although there was no significant difference (Fig. S1). The beta diversity analysis showed significant structure of microbial community differences among Donor, ASD and WMT (Fig. 3B). The genus level of intestinal flora composition of the ASD patients and the health donor was different. After WMT, the gut microbiota of ASD children were significantly changed, such as the abundance of *Bacteroides* decreased while *Prevotella_9*, *Bifidobacterium* increased (Fig. 3C and D, Table S1). We calculated the Bray–Curtis distance of ASD children's OUT between different times of WMT,

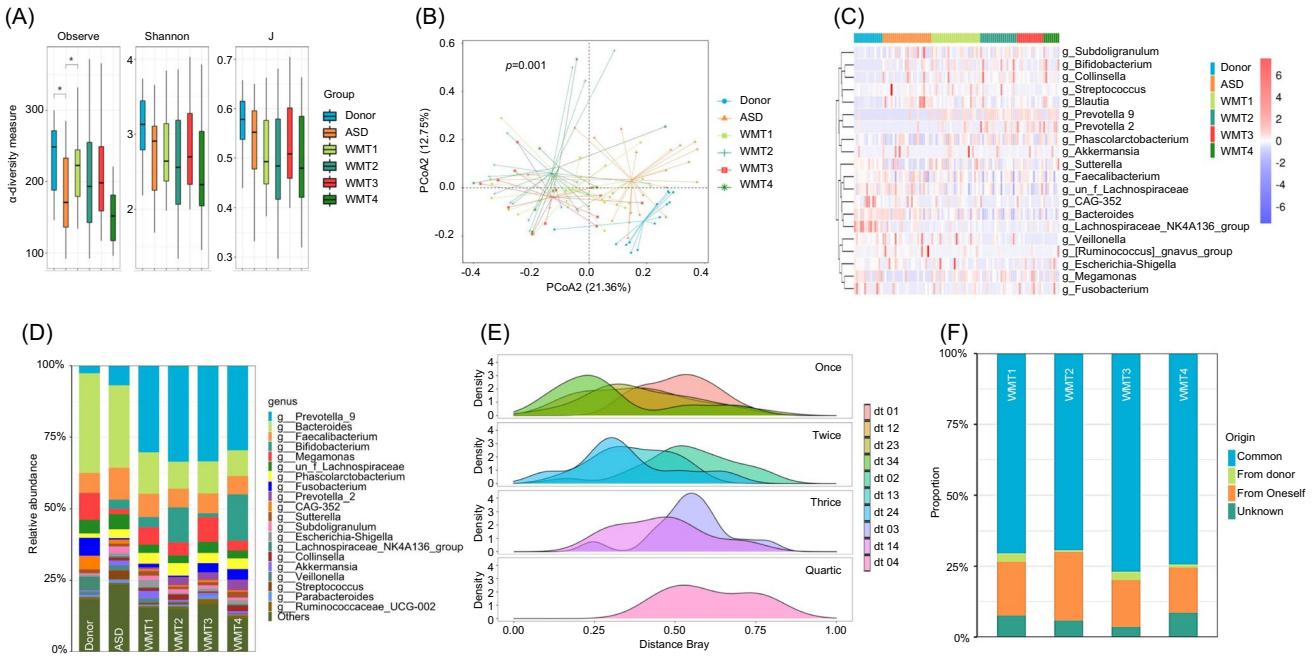


Fig. 3 Changes in gut microbiota after WMT treatment. **A** Alpha diversity analysis, including Observe, Shannon, and J analysis for the indicated groups. **B** Beta diversity analysis for the indicated groups. **C** Abundance expression levels of the top 20 genera were shown as individuals during the WMT treatment. **D** Abundance expression levels of the top 20 genera were shown as time node during the WMT treatment. **E** 16S reads were clustered into OTUs, and the OTU distance was used to estimate diversity after WMT treatments: "dt" means distance, there were four group (dt01, dt12, dt23, and dt34) significantly changed at the end of the first WMT course, the number were three (dt02, dt13, and dt24) at the end of the second WMT course, the number were two (dt03 and dt14) at the end of the third WMT course, and there was only one (dt04) at the end of the fourth WMT course. **F** Chasing the origin of the gut microbiota at the genus level after each WMT treatment. n ≧ 8, $*p < 0.05$

and then found that the OTU changed significantly at WMT-1, and the changes in the next WMT was always less than that in the former one (Fig. 3E). After each WMT, we explored the genera-level origin of ASD children, and then found that 1.07% of the genera came from the donor after WMT-4 (Fig. 3F).

Chasing Gut Bacterial Genus Change During WMT Treatment

Then, we performed the paired-rank sum test difference analysis at the genus level, and then found 44 significant different genera. After WMT, the richness of 20 genera increased significantly and the richness of 24 genera decreased significantly (Fig. 4A). The abundances of these 44 genera in the sample are shown in the Fig. 4B. We found that in the process of WMT, the abundance of *Family_XIII_UCG-001*, *GCA-900066575*, *Prevotella_2*, *Prevotella_9*, *Ruminococcaceae_UCG-009*, and *un_f_Prevotellaceae* had a stable increase trend, while *Bacteroidetes*, *Klebsiella*, *Parasutterella*, *Romboutsia*, *un_f_Peptostreptococcaceae*, *Veillonella* had a stable decline trend (Fig. 4C and D).

Speculate the Underlying Genus Functional Pathways During WMT Treatment

We next analyze the correlation of 44 genera between ASD and WMT. *Bacteroides* and *Prevotella_9* had a strong negative correlation, while *gnavus_group* positively and significantly associated with *Erysipelatoclostridium* and *innocuum_group*. *Lachnospiraceae_NC2004_group* positively and significantly associated with *Ruminococcaceae_UCG-002*. *innocuum_group* also positively correlated with *Erysipelatoclostridium* (Fig. 5A). we then analyze the relationship of ABC score, DAO, D-lactate, Endotoxin, sleeping disorder, Intestinal lesion, Bristol with the method of Pearson coefficients, and found that 67 genera were significantly correlated (Fig. 5B, Table S2). Sixteen of them showed significant differences in nonparametric Kruskal–Wallis rank sum test analysis, among them, *ylanophilum_group*, *Bacteroides*, *Flavonifractor*, *Intestinimonas*, *Parasutterella* were significantly positively correlated with ABC score, while *Allisonella*, *Odoribacter*, *un_f_Prevotellaceae* were significantly negatively correlated with ABC score (Table S3). Moreover, 63 functional pathways were found to be significantly correlated with clinical indicators (Table S4). There were significant strong correlations between 24 genera and

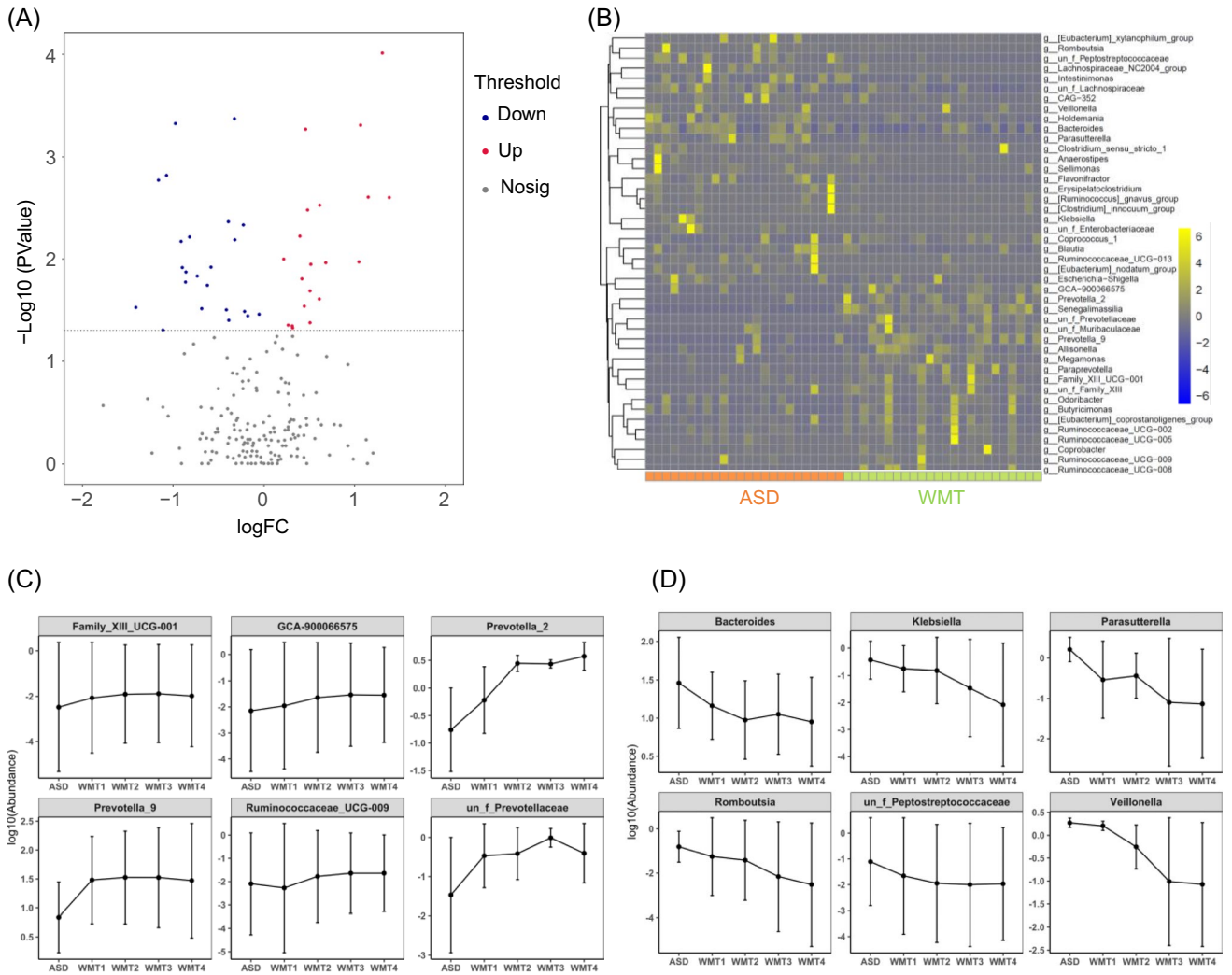


Fig. 4 Profile different genera during WMT treatment. **A** Kruskal–Wallis rank sum test of pre-WMT and post-WMT at the genus level. **B** There were 44 genera significantly changed after WMT treatments. **C** The stable increasing trend genera during WMT treatment. **D** The stable decline trend genera during WMT treatment. n ≧ 8

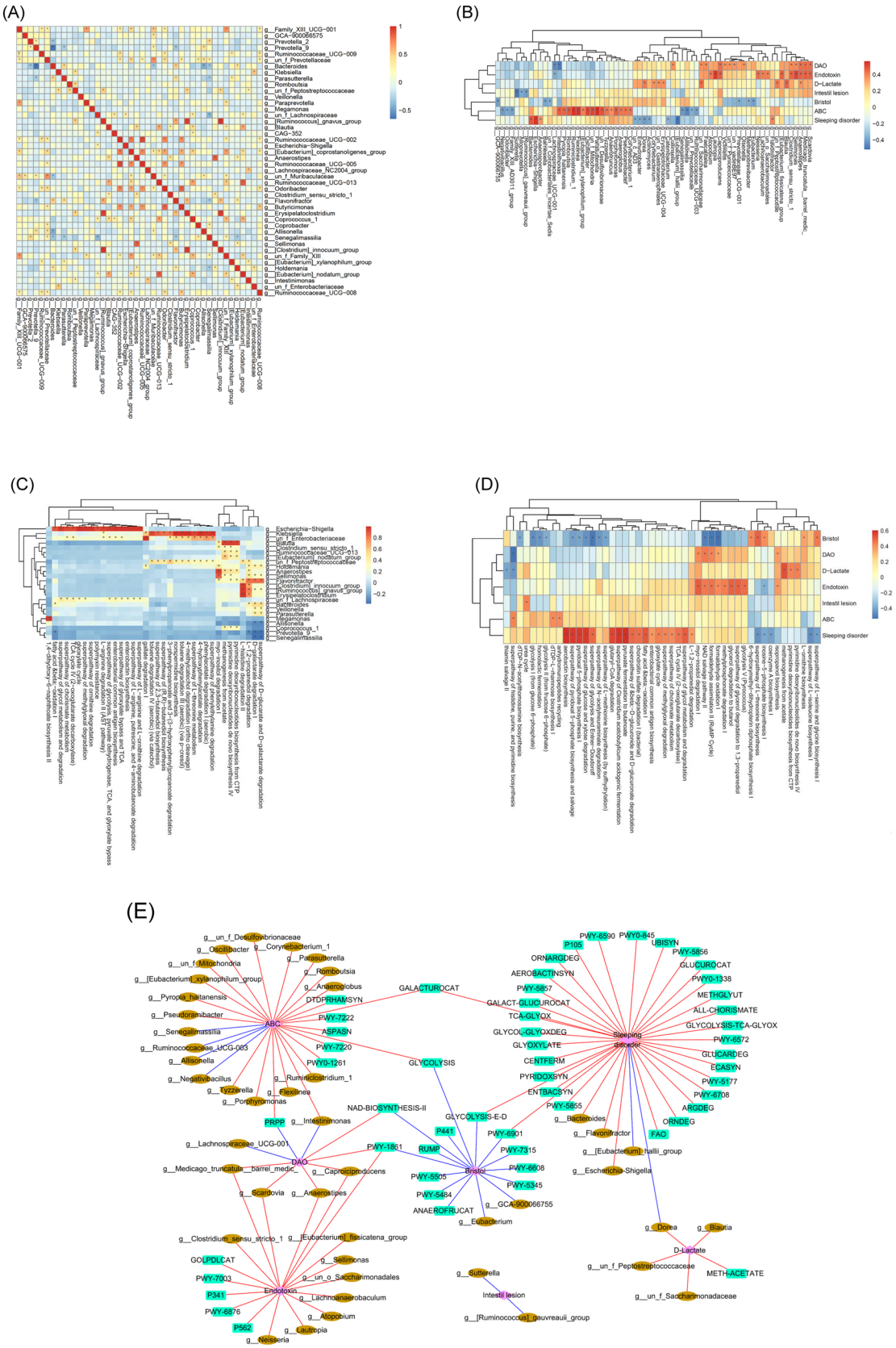
36 pathways (Fig. 5C). We selected genera and pathways that were significantly correlated with clinical indicators ($p < 0.05$, $|r| > 0.4$), and performed a network interaction analysis with clinical indicators to find deeper relationships (Fig. 5D and E).

Discussion

In the present study, we found that WMT improves constipation and sleeping quality in ASD children. In addition, gut dysbiosis such as upregulation of *Bacteroides/Flavonifractor/Parasutterella*, downregulation of *Prevotella_9*, was correlated with sleeping disorder of ASD children. Our results further suggested that WMT targeted and reversed the richness of these differential genera to decrease toxic metabolic production and improve detoxification, which would in turn ameliorate ASD and its symptom of sleeping disorder (Fig. 6).

It's very important to obtain the good relations of cooperation from young ASD children. During the WMT process, we strongly recommended that these children should be accompanied by the closest and most trusted person(s), and do what they are most interested in, such as playing favorite toys (or the other objects that make them feel safe and calm), reading favorite fairy tales, and/or watching favorite cartoons. Besides, we performed TET tube by colonoscopy. The reason is that the lumen of the ileocecal region was thicker and larger, have more mucosal folds, less Food residue accumulation while compared to small intestine.

Previous study had suggested that sleeping disorder in ASD children would aggravate the main symptoms of ASD


(A)
(B)
DAO
Endotoxin
D−Lactate
Intestil lesion
Bristol
ABC
Sleeping disorder
(C)
(D)
(E)
Sleeping disorder
Bristol
GLYCOLYSIS
GALACTURONATE
ABC
DAO
Endotoxin
NAD-BIOSYNTHESIS-II
GLYCOLYSIS-E-D
D-Lactate
Intestil lesion
METH-ACETATE

◂**Fig. 5** The relationship of WMT-modified gut bacterial species and ASD symptoms. **A** Correlation analysis of significantly different genera after WMT treatment. **B** Correlation analysis of the significantly different genera and clinical indicators. **C** Functional pathway analysis of the significantly different genera. **D** Correlation analysis of the significantly different genera related functional pathways and clinical indicators. **E** Clinical indicators interact with a network of the significantly different genera related pathways, the red/blue line represents a significant positive/negative correlation. $n \geqq 8$, $*p < 0.05$

(Hua et al., 2020). In line with this, our results showed that WMT had significant effects in ameliorating ASD children by improving constipation and sleep quality, while there was not any not any adverse event reported. The efficacy and safety of WMT might largely depend on the quality of microbiota. Actually, in the process of health microbiota preparation, feces-derived viruses and pro-inflammatory metabolites were removed during the washing process to eliminate the potential adverse events (Marcella et al., 2021; Zhang et al., 2020c). Besides, as described above, we only used the fresh microbiota in the present study, all processes together of health microbiota preparation and transplantation were within one hour to maintain its freshness and vitality as much as possible. Otherwise, previous studies showed that FMT would ameliorate intestinal barrier dysfunction in several animal models, but so far there was not enough directly and sufficient evidence in a human study (Craven et al., 2020; Li et al., 2015; Prochazkova et al., 2019; Rao et al., 2021). Our results here showed that WMT did not observed the significant changes of DAO and D-lactate in the selected population. Thus, it would be better to further recruit a new and independent cohort to validate the clinical relevance of WMT in regulating the integrity and function of the intestinal barrier of ASD patients.

We next characterized the microbiota profile in ASD children before and after WMT treatment. Our results here showed that the first WMT intervention significantly increased the richness of the gut microbiota in ASD children, although no continuous change was found in the subsequent WMT intervention. These results were in line with Bray–Curtis distance analysis, we found that OTU was significantly changed at the first WMT intervention, and the changes in the next WMT were always less than that in the former. These results suggested that WMT successfully changed the microbial richness and community structure. Moreover, this change tended to remain stable and continuous.

Besides, there was a strong relationship between ASD remission and improved sleep during WMT treatment. Our results here showed that several genera were significantly correlated with sleeping disorder of ASD children. Among these genera, *Bacteroides* and *Flavonifractor* had a positive correlation, both positively correlated with the sleeping disorder of ASD children, they also significantly and positively correlated with D-glucarate degradation and/or myo-inositol degradation. There was no significant correlation between *Parasutterella* and sleeping disorder of ASD children, though it's positive. However, not only did *Parasutterella* and *Bacteroides/Flavonifractor* have a positive correlation, but also significantly and positively correlated with D-glucarate degradation. Otherwise, *Bacteroides* and *Prevotella_9* had a negative correlation, they had contrary effects on the sleeping disorder of ASD children and D-glucarate (D-glucaric acid salt) degradation. Indeed, 23 pathways were dysfunction and involved in sleeping disorder of ASD children. Among these pathways, the super-pathway of glycolysis/Entner−Doudoroff/D-glucuronide degradation was significantly and positively correlated with the sleeping disorder of ASD children. These pathways were mainly involved in the synthetic pathway of glucaric acid (Entner & Doudoroff, 1952; Moon et al., 2009). These results indicated that upregulation of *Bacteroides/Flavonifractor/Parasutterella*, downregulation of *Prevotella_9* would decrease production of glucaric acid in the sleeping disorder of ASD children. Previous studies have highlighted that glucaric acid played an important role in detoxification and protection by inhibiting toxic chemical pathogenesis and inhibiting beta-glucuronidase activity, at least including cholesterol reduction, inflammation suppression, and cancer prevention (Walaszek et al., 1996; Zółtaszek et al., 2008). Thus, these results indicated that WMT treatment would ameliorate sleeping disorder of ASD children by significantly upregulating *Prevotella_9* while significantly downregulating *Bacteroides/Flavonifractor/Parasutterella* to increase glucaric acid production and its salts.

Interestingly, *Bacteroides* and *Prevotella_9* also had a significant and contrary effect on fatty acid β-oxidation pathway, and this pathway also significantly and positively correlated with sleeping disorder of ASD children. A previous study showed that upregulation of short-chain fatty acid beta-oxidation promoted theta frequency generation during paradoxical sleep (Tafti et al., 2003). Thus, our results also suggested that upregulation of *Bacteroides* and/or downregulation of *Prevotella_9* could increase the frequency to trigger abnormal wake-up, which in turn contributed to sleeping disorder of ASD children, and this metabolic disorder appeared to have the benefit of WMT treatment. We also found that *Bacteroides* and *Flavonifractor* significantly promoted degradation of L-1,2-propanediol (propylene glycol) in the sleeping disorder of ASD children, while *Prevotella_9* had a contrary effect. Previous studies showed that degradation of L-1,2-propanediol would generate toxic metabolites, such as aldehydes, contributing to respiratory diseases and diabetic retinopathy, and monoamine oxidase inhibitors ameliorate diseases associated with the central nervous system in part by decreasing aldehyde generation (Farsalinos & Polosa, 2014; Murata et al., 2020; Ostadkarampour &

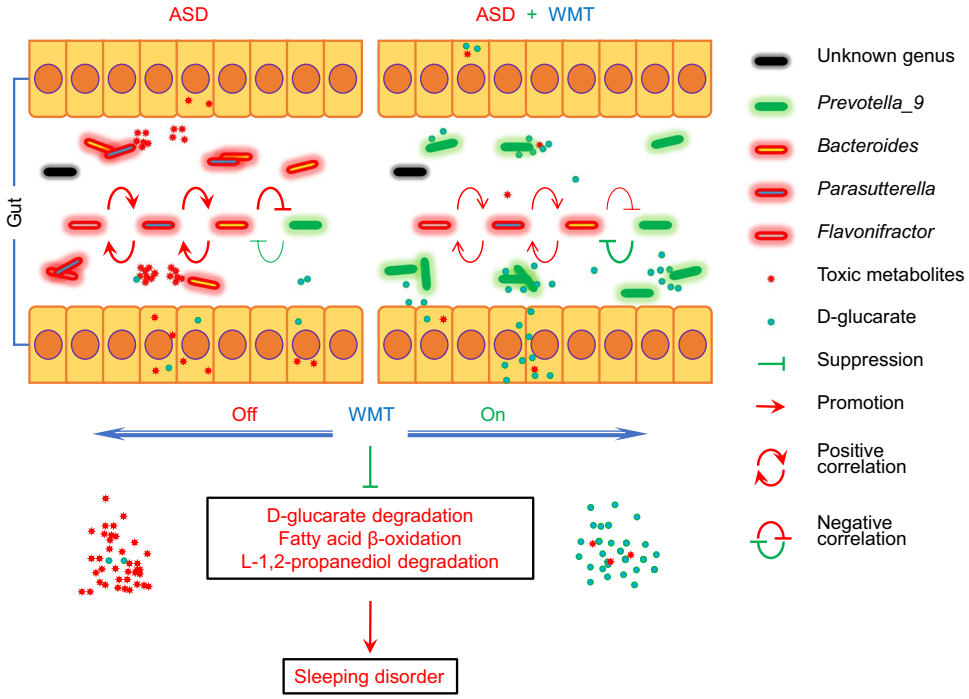


Fig. 6 Model of the role of WMT in ameliorating the sleeping disorder of ASD patients. *Parasutterella* was significantly and positively correlated with *Bacteroides/Flavonifractor*, while *Bacteroides* was significantly and negatively correlated with *Prevotella_9* in ASD patients. WMT upregulated *Prevotella_9*, while downregulated *Bacteroides/Flavonifractor/Parasutterella* to decrease toxic metabolic production and improve detoxification, which in turn ameliorated ASD and its sleeping disorder symptom

Putnins, 2021). Taken these together, our results suggested that WMT could target *Bacteroides/Flavonifractor/Prevotella_9* to decrease the production of toxic metabolites, which in turn ameliorate the sleeping disorder of ASD children. However, more evidence for correlation of these metabolic disorders and sleeping disorder of ASD children should be addressed in future.

Our next found correlations of another clinical indicator and the ABC score were changed during WMT treatment. There were 68% of ASD children who suffered constipation, but not diarrhea (data not shown). WMT significantly improved constipation of ASD children, it also tended to decrease the correlation coefficient of the Bristol score and the ABC score, although the correlation did no show statistical difference. Besides, there were six genera significantly and negatively correlated with Bristol score, but no one was significant changed by WMT treatment. WMT tended to ameliorate intestinal lesion and decrease the correlation coefficient of the intestinal lesion score and the ABC score, but both did not show statistical difference. WMT also tended to ameliorate intestinal barrier dysfunction and increase the correlation coefficient of ABC score and DAO/D-lactate/Endotoxin respectively, but all of which did not show statistical difference. These results tended to suggest that WMT would ameliorate constipation and intestinal barrier dysfunction of ASD children, which in turn inhibited the entry of toxic metabolites into the gut–brain axis and promoted their emission. Therefore, it rationally prompts us to collect more samples to confirm these results in the future study.

Conclusion

Taken these together, our data provided directly evidences to support that WMT significantly improved the behavioral and sleeping disorder symptoms of ASD children. Mechanistically, WMT stably and continuously changed the profile of ASD-related bacteria, especially upregulated *Bacteroides/Flavonifractor/Parasutterella*, downregulated *Prevotella_9*, which would decrease toxic metabolic production and improve detoxification to ameliorate ASD and its symptoms.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12275-023-00069-x.

Acknowledgements We gratefully thank all enrolled health donors, enrolled ASD children and their families for participating in this study. We then do appreciate all funders who have provided the financial supports for this study.

This study was supported by Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (ZYYCXTD-C-202208), Guangdong Provincial Key Laboratory of TCM Pathgenesis and Prescriptions of Heart and Spleen Diseases (2022B1212010012), Special Project for Research and Development in Key areas of Guangdong Province (2020B1111100011), the Natural Science Foundation of Guangdong Province (2018A030313639, 2019A1515010125, 2023A1515010751), Guangdong Key Discipline Research Project of Department of Education of Guangdong Province (2019-GDXK-0013), and COVID-19 Epidemic Prevention and Control Special Research Project of Department of Education of Guangdong Province (2020KZDZX1132). Basic and Applied Basic Research Project of Guangzhou Basic Research Program (202201010134), Discipline Collaborative Innovation Team of Guangzhou University of Traditional Chinese Medicine (2021xk36). All funders had no role in

the design of the study and collection, analysis, interpretation of data and in writing the manuscript, the decision to submit the manuscript for publication.

Author Contributions X-XH and H-FP conceived and designed the study. N-HL, H-QL, J-YZ, M-LZ, and L-HW carried out sample collection 340 and washed microbiota transplantation. N-HL and J-YZ performed data analyses. N-HL and H-QL prepared the manuscript draft, X-XH and H-FP revised the manuscripts. X-XH submitted 342 the manuscript. All authors have read and approved the final manuscript.

Data Availability The datasets of 16S rRNA gene sequencing data presented in this study can be found in the NCBI Sequence Read Archive (SRA) (accession number: PRJNA744027).

Declarations

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval and Consent to Participate The studies involving human participants were reviewed and approved by The Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University (Approval License No. YLS2020-14). Written informed consent to participate in this study was provided by the participants' parents for the publication of data included in this article.

Consent for Publication Not applicable.

References

American Psychiatric Association, DSM-5 Task Force. (2013). *Diagnostic and statistical manual of mental disorders DSM-5™* (5th ed.). American Psychiatric Publishing, Inc. https://doi.org/10.1176/appi.books.9780890425596

Baio, J., Wiggins, L., Christensen, D. L., Maenner, M. J., Daniels, J., Warren, Z., Kurzius-Spencer, M., Zahorodny, W., Robinson Rosenberg, C., White, T., et al. (2018). Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 Sites, United States, 2014. *Morbidity and Mortality Weekly Report. Surveillance Summaries, 67*, 1–23.

Bruni, O., Ottaviano, S., Guidetti, V., Romoli, M., Innocenzi, M., Cortesi, F., & Giannotti, F. (1996). The Sleep Disturbance Scale for Children (SDSC). Construction and validation of an instrument to evaluate sleep disturbances in childhood and adolescence. *Journal of Sleep Research, 5*, 251–261.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*, 335–336.

Cerminara, M., Spirito, G., Pisciotta, L., Squillario, M., Servetti, M., Divizia, M. T., Lerone, M., Berloco, B., Boeri, S., Nobili, L., et al. (2021). Case report: whole exome sequencing revealed disease-causing variants in two genes in a patient with autism spectrum disorder, intellectual disability, hyperactivity, sleep and gastrointestinal disturbances. *Frontiers in Genetics, 12*, 625564.

Christensen, D. L., Braun, K. V. N., Baio, J., Bilder, D., Charles, J., Constantino, J. N., Daniels, J., Durkin, M. S., Fitzgerald, R. T., Kurzius-Spencer, M., et al. (2018). Prevalence and characteristics of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2012. *Morbidity and Mortality Weekly Report. Surveillance Summaries, 65*, 1–23.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., & Tiedje, J. M. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research, 42*, D633–D642.

Craven, L., Rahman, A., Nair Parvathy, S., Beaton, M., Silverman, J., Qumosani, K., Hramiak, I., Hegele, R., Joy, T., Meddings, J., et al. (2020). Allogenic fecal microbiota transplantation in patients with nonalcoholic fatty liver disease improves abnormal small intestinal permeability: a randomized control trial. *The American Journal of Gastroenterology, 115*, 1055–1065.

Cui, B., Feng, Q., Wang, H., Wang, M., Peng, Z., Li, P., Huang, G., Liu, Z., Wu, P., Fan, Z., et al. (2015). Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: safety, feasibility, and efficacy trial results. *Journal of Gastroenterology and Hepatology, 30*, 51–58.

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., Huttenhower, C., & Langille, M. G. I. (2020). PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology, 38*, 685–688.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*, 2460–2461.

Entner, N., & Doudoroff, M. (1952). Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. *The Journal of Biological Chemistry, 196*, 853–862.

Farmer, C., & Aman, M. G. (2020). Aberrant behavior checklist. In F. Volkmar (Ed.), *Encyclopedia of autism spectrum disorders*. USA: Springer, New York.

Farsalinos, K. E., & Polosa, R. (2014). Safety evaluation and risk assessment of electronic cigarettes as tobacco cigarette substitutes: a systematic review. *Therapeutic Advances in Drug Safety, 5*, 67–86.

Fecal Microbiota Transplantation-standardization Study Group. (2020). Nanjing consensus on methodology of washed microbiota transplantation. *Chinese Medical Journal, 133*, 2330–2332.

Ghosn, F., Navalón, P., Pina-Camacho, L., Almansa, B., Sahuquillo-Leal, R., Moreno-Giménez, A., Diago, V., Vento, M., & García-Blanco, A. (2022). Early signs of autism in infants whose mothers suffered from a threatened preterm labour: a 30-month prospective follow-up study. *European Child & Adolescent Psychiatry, 31*, 1–13.

Hua, X., Zhu, J., Yang, T., Guo, M., Li, Q., Chen, J., & Li, T. (2020). The gut microbiota and associated metabolites are altered in sleep disorder of children with autism spectrum disorders. *Frontiers in Psychiatry, 11*, 855.

Hunter, P. (2023). Autism therapy at crossroads. *EMBO Reports, 24*, e56915.

Kang, D. W., Adams, J. B., Gregory, A. C., Borody, T., Chittick, L., Fasano, A., Khoruts, A., Geis, E., Maldonado, J., McDonough-Means, S., et al. (2017). Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome, 5*, 10.

Keshtkarjahromi, M., Palvadi, K., Shah, A., Dempsey, K. R., & Tonarelli, S. (2021). Psychosis and catatonia in fragile X syndrome. *Cureus, 13*, e12843.

Kramer, I., Lipkin, P. H., Marvin, A. R., & Law, P. A. (2015). A genetic multimutation model of autism spectrum disorder fits disparate twin concordance data from the USA and Canada. *International Scholarly Research Notices, 2015*, 519828.

Lewis, S. J., & Heaton, K. W. (1997). Stool form scale as a useful guide to intestinal transit time. *Scandinavian Journal of Gastroenterology, 32*, 920–924.

Li, M., Liang, P., Li, Z., Wang, Y., Zhang, G., Gao, H., Wen, S., & Tang, L. (2015). Fecal microbiota transplantation and bacterial

consortium transplantation have comparable effects on the re-establishment of mucosal barrier function in mice with intestinal dysbiosis. *Frontiers in Microbiology, 6*, 692.

Liu, X. J., Wu, L. H., Xie, W. R., & He, X. X. (2020). Faecal microbiota transplantation simultaneously ameliorated patient's essential tremor and irritable bowel syndrome. *Psychogeriatrics, 20*, 796–798.

Liu, Z., Mao, X., Dan, Z., Pei, Y., Xu, R., Guo, M., Liu, K., Zhang, F., Chen, J., Su, C., et al. (2021). Gene variations in autism spectrum disorder are associated with alteration of gut microbiota, metabolites and cytokines. *Gut Microbes, 13*, 1–16.

Lynch, S. V., & Pedersen, O. (2016). The human intestinal microbiome in health and disease. *The New England Journal of Medicine, 375*, 2369–2379.

Maenner, M. J., Shaw, K. A., Baio, J., Washington, A., Patrick, M., DiRienzo, M., Christensen, D. L., Wiggins, L. D., Pettygrove, S., Andrews, J. G., et al. (2020). Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2016. *Morbidity and Mortality Weekly Report. Surveillance Summaries, 69*, 1–12.

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*, 2957–2963.

Marcella, C., Cui, B., Kelly, C. R., Ianiro, G., Cammarota, G., & Zhang, F. (2021). Systematic review: the global incidence of faecal microbiota transplantation-related adverse events from 2000 to 2020. *Alimentary Pharmacology & Therapeutics, 53*, 33–42.

Moon, T. S., Yoon, S. H., Lanza, A. M., Roy-Mayhew, J. D., & Prather, K. L. (2009). Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*. *Applied and Environmental Microbiology, 75*, 589–595.

Murata, M., Noda, K., & Ishida, S. (2020). Pathological role of unsaturated aldehyde acrolein in diabetic retinopathy. *Frontiers in Immunology, 11*, 589531.

Nitschke, A., Deonandan, R., & Konkle, A. T. (2020). The link between autism spectrum disorder and gut microbiota: a scoping review. *Autism, 24*, 1328–1344.

Ostadkarampour, M., & Putnins, E. E. (2021). Monoamine oxidase inhibitors: a review of their anti-inflammatory therapeutic potential and mechanisms of action. *Frontiers in Pharmacology, 12*, 676239.

Panzer, A. R., & Lynch, S. V. (2020). Gut microbial regulation of autism spectrum disorder symptoms. *Trends in Endocrinology and Metabolism, 31*, 809–811.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2009). FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution, 26*, 1641–1650.

Prochazkova, P., Roubalova, R., Dvorak, J., Tlaskalova-Hogenova, H., Cermakova, M., Tomasova, P., Sediva, B., Kuzma, M., Bulant, J., Bilej, M., et al. (2019). Microbiota, microbial metabolites, and barrier function in a patient with anorexia nervosa after fecal microbiota transplantation. *Microorganisms, 7*, 338.

Rao, J., Xie, R., Lin, L., Jiang, J., Du, L., Zeng, X., Li, G., Wang, C., & Qiao, Y. (2021). Fecal microbiota transplantation ameliorates gut microbiota imbalance and intestinal barrier damage in rats with stress-induced depressive-like behavior. *The European Journal of Neuroscience, 53*, 3598–3611.

Rhee, S. H., Pothoulakis, C., & Mayer, E. A. (2009). Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nature Reviews Gastroenterology & Hepatology, 6*, 306–314.

Roussin, L., Prince, N., Perez-Pardo, P., Kraneveld, A. D., Rabot, S., & Naudon, L. (2020). Role of the gut microbiota in the pathophysiology of autism spectrum disorder: clinical and preclinical evidence. *Microorganisms, 8*, 1369.

Sommer, F., Anderson, J. M., Bharti, R., Raes, J., & Rosenstiel, P. (2017). The resilience of the intestinal microbiota influences health and disease. *Nature Reviews Microbiology, 15*, 630–638.

Tafti, M., Petit, B., Chollet, D., Neidhart, E., de Bilbao, F., Kiss, J. Z., Wood, P. A., & Franken, P. (2003). Deficiency in short-chain fatty acid beta-oxidation affects theta oscillations during sleep. *Nature Genetics, 34*, 320–325.

Vendrik, K. E. W., Ooijevaar, R. E., de Jong, P. R. C., Laman, J. D., van Oosten, B. W., van Hilten, J. J., Ducarmon, Q. R., Keller, J. J., Kuijper, E. J., & Contarino, M. F. (2020). Fecal microbiota transplantation in neurological disorders. *Frontiers in Cellular and Infection Microbiology, 10*, 98.

Walaszek, Z., Szemraj, J., Hanausek, M., Adams, A. K., & Sherman, U. (1996). D-Glucaric acid content of various fruits and vegetables and cholesterol-lowering effects of dietary D-glucarate in the rat. *Nutrition Research, 16*, 673–681.

Wang, B., Cao, F., & Boyland, J. T. (2019). Addressing autism spectrum disorders in China. *New Directions for Child and Adolescent Development, 163*, 137–162.

Xie, W. R., Yang, X. Y., Xia, H. H., Wu, L. H., & He, X. X. (2019). Hair regrowth following fecal microbiota transplantation in an elderly patient with alopecia areata: a case report and review of the literature. *World Journal of Clinical Cases, 7*, 3074–3081.

Ye, Z. N., Xia, H. H., Zhang, R., Li, L., Wu, L. H., Liu, X. J., Xie, W. R., & He, X. X. (2020). The efficacy of washed microbiota transplantation on *Helicobacter pylori* eradication: a pilot study. *Gastroenterology Research and Practice, 2020*, 8825189.

Żebrowska, P., Łaczmańska, I., & Łaczmański, Ł. (2021). Future directions in reducing gastrointestinal disorders in children with ASD using fecal microbiota transplantation. *Frontiers in Cellular and Infection Microbiology, 11*, 630052.

Zhang, M., Chu, Y., Meng, Q., Ding, R., Shi, X., Wang, Z., He, Y., Zhang, J., Liu, J., Zhang, J., Yu, J., Kang, Y., & Wang, J. (2020a). A quasi-paired cohort strategy reveals the impaired detoxifying function of microbes in the gut of autistic children. *Science Advances, 6*, eaba3760.

Zhang, T., Long, C., Cui, B., Buch, H., Wen, Q., Li, Q., Ding, X., Ji, G., & Zhang, F. (2020b). Colonic transendoscopic tube-delivered enteral therapy (with video): a prospective study. *BMC Gastroenterology, 20*, 135.

Zhang, T., Lu, G., Zhao, Z., Liu, Y., Shen, Q., Li, P., Chen, Y., Yin, H., Wang, H., Marcella, C., et al. (2020c). Washed microbiota transplantation vs. manual fecal microbiota transplantation: clinical findings, animal studies and in vitro screening. *Protein & Cell, 11*, 251–266.

Zheng, Y. M., Chen, X. Y., Cai, J. Y., Yuan, Y., Xie, W. R., Xu, J. T., Xia, H. H., Zhang, M., He, X. X., & Wu, L. H. (2021). Washed microbiota transplantation reduces proton pump inhibitor dependency in nonerosive reflux disease. *World Journal of Gastroenterology, 27*, 513–522.

Zhong, H. J., Zeng, H. L., Cai, Y. L., Zhuang, Y. P., Liou, Y. L., Wu, Q., & He, X. X. (2021). Washed microbiota transplantation lowers blood pressure in patients with hypertension. *Frontiers in Cellular and Infection Microbiology, 11*, 679624.

Zhou, H., Xu, X., Yan, W., Zou, X., Wu, L., Luo, X., Li, T., Huang, Y., Guan, H., Chen, X., et al. (2020). Prevalence of autism spectrum disorder in China: a nationwide multi-center population-based study among children aged 6 to 12 years. *Neuroscience Bulletin, 36*, 961–971.

Zółtaszek, R., Hanausek, M., Kiliańska, Z. M., & Walaszek, Z. (2008). The biological role of D-glucaric acid and its derivatives: potential use in medicine. *Advances in Hygiene and Experimental Medicine, 62*, 451–462.