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Effects of Tuina targeting different body parts on the behaviors and gut microflora of autistic spectrum disorder rat models

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ABSTRACT

Objective To investigate the effects of Tuina targeting different body parts on the behaviors and gut microflora of rat models with valproic acid (VPA)-induced autistic spectrum disorder (ASD).

Methods Twenty female Sprague Dawley (SD) rats with 12.5 d of pregnancy were randomly divided into VPA model group [intraperitoneal injection of VPA (600 mg/kg), n = 15] and saline group (intraperitoneal injection of equal amount of normal saline, n = 5). The offspring male rats injected with saline were secleted as control group. The offspring male rats injected with VPA were randomly divided into VPA, dorsal, and abdominal groups (n = 7 in each group). On the 21st day after birth, three-chamber social test, open field test, and marble-burying test were carried out to observe the social abilities, anxiety behaviors, and stereotypical behaviors of rats in the four groups. Rats in dorsal and abdominal groups underwent Tuina for 14 d, twice a day. On the 35th day, behavioral tests were conducted again, and intestinal contents were taken for species composition and structural analysis, as well as marker and differential species analysis.

Results (i) According to behavioral observations, compared with VPA group, the social and movement time in the central open field of rats in dorsal group increased significantly (P < 0.05), and the number of buried marbles decreased markedly (P < 0.01), indicating improvement on their social abilities, anxiety behaviors, and stereotypical behaviors as consequences of dorsal Tuina; and the number of buried marbles was reduced as well in abdominal group when compared with VPA group (P < 0.05), suggesting the improvement on their stereotypical behaviors following abdominal Tuina. In the marble-burying test, the number of marbles buried in dorsal group was less than in abdominal group, and the stereotypical behaviors were improved more significantly (P < 0.05), and there were no significant differences in the three-chamber social and open field tests between the two groups (P >0.05). (ii) In accordance with intestinal microflora detection results, compared with VPA group, both dorsal and abdominal groups showed increased richness (P < 0.05) and elevated diversity (P < 0.05 in dorsal group and P < 0.01 in abdominal group) in intestinal microflora. The results of differential analysis indicated that at the phylum level, compared with VPA group, the relative abundance of *Firmicutes* in rats in abdominal group showed a significant reduction trend (P < 0.05); at the genus level, compared with VPA group, the relative abundance of *Lactobacillus* in

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rats in dorsal and abdominal groups decreased significantly ($P < 0.05$). Dorsal group also showed significant increase in the genus *Blautia* in the analysis of marker species compared with VPA group ($P < 0.05$).

Conclusion Tuina impacted the behavior and gut microflora structure of ASD model rats. Dorsal intervention had a significant effect on social abilities, anxiety behaviors, and stereotypical behaviors of ASD model rats, while abdominal intervention only had an obvious effect on stereotypical behaviors. Both dorsal and abdominal interventions increased the richness and diversity of gut microflora of ASD model rats, with abdominal intervention improving the intestinal microbial diversity more significantly and resulting in a more uniform species distribution.

1 Introduction

Autistic spectrum disorder (ASD) is a group of neurodevelopmental disorders characterized by limited social interactions and communications, along with repetitive, stereotypical behaviors, and narrow interests [1]. The causes of ASD are thought to be closely associated with external environmental factors, such as maternal diseases, teratogenic influences in early pregnancy, and perinatal injuries, as well as genetic factors, and intestinal microecological disorders [2]. The concept of the microbiota-gut-brain axis (MGBA) is based on the fact that gut microorganisms affect the gut and the central nervous system through neurological, immunological, and endocrine pathways, resulting in a two-way communication between the gut and the brain [3]. Gut microbes affect brain functions and behaviors through MGBA, which are all closely related to ASD development [4]. As main participants, gut microfloras regulate the host's brain functions and behaviors by influencing the MGBA, so changes in the gut microflora can affect the development and function of the central nervous system through the axis [5]. Imbalanced gut microflora has the potential to trigger inflammation, gastrointestinal discomfort, and an increase in neurotoxic metabolites, leading to abnormal stress responses, impaired cognitive abilities, and the manifestation of anxiety and depression, possibly contributing to the causes of ASD [6]. Clinical studies have confirmed differences in the fecal microbiota between normal individuals and ASD patients [7]. Bloating, diarrhea, and constipation are common gastrointestinal symptoms in ASD patients which are positively correlated with the health conditions of patients [8]. These symptoms also exacerbate behavioral problems, such as stereotypical behaviors, social impairment, and hyperactivity. When the gastrointestinal symptoms improve, patients' ASD symptoms could be reduced accordingly [9, 10]. Therefore, gut microbes may serve as a viable target for ASD intervention.

In recent years, the increasing prevalence of ASD has received growing attention, promoting the utilization of traditional Chinese medicine (TCM) for early intervention in ASD patients [11]. Tuina is an effective and affordable intervention that applies stimulation to the body surface for health and therapeutic purposes. Researchers studied the scalp electroencephalogram (EEG) of 33 ASD children aged 3 to 6 years undergoing passive fingertip tactile stimulation, some of whom had cognitive or language impairments. The study found that the accessibility measures of tactile cortex processing might be an indicator of tactile response in ASD children, and their EEG levels might be altered by cortical stimulation through Tuina [12]. It was reported that Tuina alleviated core behaviors and touch aversion in ASD children, possibly due to enhanced parasympathetic nerve activity [13]. It was demonstrated that Tuina effectively promoted the colonization of intestinal probiotics in children, regulated intestinal microecological balance, and restored intestinal functions [14]. The abdominal and dorsal parts were the main parts of Tuina for ASD patients [15, 16], through which core ASD symptoms were alleviated.

ASD rat models and ASD patients are similar in clinical manifestations, rendering them ideal study subjects [17]. It was reported that maternal exposure to valproic acid (VPA) during pregnancy could easily induce fetal ASD [18], and an ASD infant rat model could be established by intraperitoneal injection of VPA on day 12.5 of maternal pregnancy [19]. This study investigated the effects of Tuina targeting different body parts on the behaviors of infant ASD rat models, including socialization, anxiety, and stereotypical behavior, as well as its effects on intestinal microorganisms *in vivo*. The possible correlation between the changes in intestinal microorganisms and behaviors in infant ASD rats was explored to provide a scientific basis for the application of TCM external therapies to treat ASD.

2 Materials and methods

2.1 Animals

A total of 20 special pathogen free (SPF) female Sprague Dawley (SD) rats that had been pregnant for 12.5 d were purchased from Hunan Slack Jingda Experimental Animal

Co., Ltd. [experimental animal license number: SCXK (Xiang) 2019-0004 and facility license number: SYXK (Xiang) 2019-0009]. All animal experiments were conducted in the Animal Experimental Center of Hunan University of Chinese Medicine in an SPF environment. All rats had ad libitum access to water and food in a standardized environment (24 – 26 °C, 50% – 70% relative humidity, and a 12 h light/dark cycle). The protocol was approved by the Animal Ethics Committee of Hunan University of Chinese Medicine (LL2022042001).

2.2 Reagents and instruments

Reagents: VPA (MedChemExpress, USA); 0.9% sodium chloride (Dezhou Jingxin Pharmaceutical, China); Q5® high-fidelity DNA polymerase (New England Biolabs, China); Quant-iT PicoGreen dsDNA assay kit and agarose gel reagent (Invitrogen, China); marker and agarose gel electrophoresis buffer (Takara, China).

Instruments: animal behavior tracing and analysis software (Beijing Zhongshi Technology, Labmaze 3.0); three-chamber social test equipment (Beijing Zhongshi Technology, ZS-SXJ-II); open field test equipment (Beijing Zhongshi Technology, ZS-LabMaze-III); polymerase chain reaction (PCR) amplification instrument (ABI, 2720); enzyme-linked immunosorbent assay (BioTek, FLX800T); electrophoresis apparatus (Beijing Liuyi Instrument Factory, DYY-6C); gel imaging system [Beijing Baijing Company, BG-gdsAUTO (130)]; homemade pushing method stimulator (Figure 1) , which employed unidirectional friction to evenly apply force to the surface of the subject's body. The input parameters on the control panel of the stimulator included distance (50 – 250 mm), pressure (50 – 1 000 g), time (1 – 99 min), frequency (1 – 200 times/min), pressing speed (1 – 999 mm/s), manual speed (1 – 999 mm/s), and lifting height (0 – 50 mm).

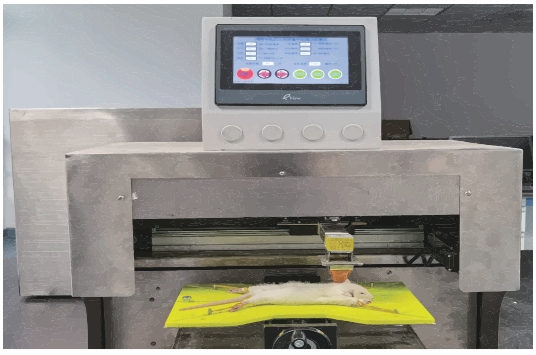

Figure 1 Homemade pushing method stimulator

2.3 ASD animal induction and allocation

The VPA model group consisted of 15 pregnant rats. ASD infant models were created by administering intraperitoneal injections of VPA (600 mg/kg) to pregnant rats with gestation of 12.5 d according to the method of SCHNEIDER's approach [20]. The saline group consisted of five pregnant rats, which received an equal amount of saline by intraperitoneal injection in proportion to their body weight. The birth of the rats was recorded as day 1. Initial behaviors were observed and measured on day 21. The modeling evaluation was determined by the three-chamber social test, in which the social time between the experimental rats and the unfamiliar rats was recorded. The success of the modeling was determined by observing whether the experimental rat exhibited social disorders or social novelty disorders. The social barriers of the three-chamber social test are as follows: in phase one, the experimental rat stayed in the empty cage for a long time with little or no contact with the unfamiliar rat (Stranger 1), suggests social barriers; in phase two, the experimental rat had more contact with Stranger 1 than with new rat (Stranger 2), indicating social novelty disorder. After the completion of modeling, 21 male offspring from VPA model group were randomly selected and divided into VPA, dorsal, and abdominal groups (n = 7). In addition, seven male offspring of saline group were selected as control group.

2.4 Body part selection and Tuina scheme

According to a study correlating age between experimental animals and humans, SD rats were observed to have entered the period at weaning on day 21 and reached puberty on day 38 [21], and because of the emphasis on early intervention in ASD, the initial behavioral levels of rats in the four groups were detected and intervened from day 21. Rats in control and VPA groups were exempted from Tuina. In abdominal group, rats experienced direct abdominal thrusts in a top-down direction, spanning from the inferior glabella to the superior pubic symphysis, with a width of 0.8 cm. Meanwhile, rats in dorsal group received direct pushing along the Du Meridian (DU), moving from the caudal root along the posterior median line to the Baihui [22], with a width of 0.8 cm. Rats in dorsal and abdominal groups were fastened to a retractable stimulator chassis using a leather rope while awake, and they were subjected to intervention with the use of a custom-made pushing method stimulator. Rats in dorsal and abdominal groups were placed in either prone or supine position, experiencing stimulation at a frequency of 100 times/min and a pressure of 100 g. Each session was performed for 20 min, twice a day, and the interval between each intervention was 6 h, for a total of 14 d. Subsequent behavioral tests [23] were performed on day 35, and intestinal microflora samples were collected for analysis.

2.5 Three-chamber social test

The three-chamber social test apparatus, measuring 60 cm × 40 cm × 40 cm, consisted of three rectangular black chambers, each with a size of 20 cm × 40 cm × 40 cm. A channel in each divider connected the three chambers. In the center of both the left and right chambers, a black cylindrical metal cage of identical size was positioned. A video monitor situated above the experimental setup, was employed to record the activities of the experimental rats in the three chambers.

During the acclimatization phase, the experimental rats were placed in the central chamber of the three chambers, allowing them 10 min to become fully acquainted with their surroundings. In phase one, Stranger 1 was positioned in metal cage 1 of the left chamber, with metal cage 2 in the right chamber left empty, enabling the experimental rat to explore freely for 10 min. In phase two, Stranger 2 was introduced into cage 2 in the right chamber, and the experimental rats were allowed 10 min for free exploration. The analysis of the time spent by the experimental rats sniffing cages 1 and 2 during the two test phases was conducted using Labmaze 3.0 behavioral software.

2.6 Open field test

The open field test apparatus consisted of a square resin box measuring 50 cm × 50 cm × 40 cm. The observation area of the experimental device was divided into 16 equal squares (4 × 4), with the central 4 squares (2 × 2) designated as the center area. To initiate the test, the experimental rats were positioned in the center of the device, and their movements were recorded by a camera for 10 min. Subsequently, Labmaze 3.0 was employed to analyze and quantify the residence time of the rats in the central area of the device.

2.7 Marble-burying test

A 5 cm thick layer of wood chips was evenly spread across the bottom of the rearing box (47 cm × 31.2 cm × 20 cm). Twelve black round marbles, each with a diameter of 1 cm, were arranged in a 4 × 3 configuration on the top of the wood chips. The experimental rats were then placed into the box, and the number of marbles that were buried by more than 70% of their volume within 30 min was recorded.

2.8 Sample collection and testing

Following the completion of behavioral tests, cervical dislocation was performed to euthanize the rats after sedation with 2% isoflurane inhalation anesthesia, and their intestinal contents were then dissected and removed. Using forceps, the rectal end of the intestine was lifted, and the intestinal contents were gently squeezed into the pre-prepared lyophilization tubes. The filled tubes were then stored at – 80 °C. All samples were analyzed using 16S genetic sequencing technology. Microbial DNA was extracted from samples and subjected to PCR amplification, library preparation, quality control, and quantification specifically targeting the V3 – V4 region. Next, the samples were differentiated using the designated tag sequences. The illumina Navaseq 6000 high-throughput sequencing platform was used to sequence the libraries that passed the test. The obtained target sequences were then filtered for quality control, and the chimeric sequences were eliminated by comparing them with the reference database, resulting in the final optimized sequences. Subsequent analyses included operational taxonomic unit (OTU) clustering analysis, species taxonomic annotation based on optimized sequences, and diversity index analysis based on the results of the OTU clustering.

2.9 Statistical analysis

All data were expressed as mean ± standard deviation (SD). SPSS 26.0 software was used for statistical analysis. GraphPad Prism 8.0 was employed to create diagrams. One-way analysis of variance (ANOVA) followed by least significant difference (LSD) posthoc test was used to examine statistical differences among groups. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Three-chamber social test

The initial phase gauged the social ability of the experimental rats by calculating the ratio of time spent sniffing the cage of Stranger 1 to the overall time spent sniffing the left and right cages. A lower ratio indicated poorer socialization ability [24]. In the subsequent phase, the social abilities of the experimental rats were evaluated by calculating the ratio of time spent sniffing the cage of Stranger 2 to the overall time spent sniffing the left and right cages, with a smaller ratio indicating a more pronounced deficit in social abilities. In phase one, the social ability of rats in dorsal group on day 35 was significantly enhanced by Tuina when compared with day 21 ($P < 0.05$). In phase two, a significant difference was observed in the social abilities deficit of rats in dorsal group after Tuina when compared with VPA group on day 35 ($P < 0.05$). There was no significant difference observed between abdominal and dorsal groups, or between abdominal and VPA groups ($P > 0.05$, Figure 2).

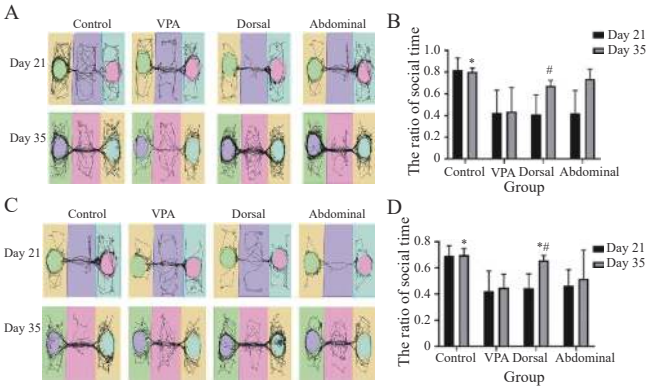

Figure 2 Three-chamber social test on day 21 and 35 ($n = 7$)

A, representative movement track of rats in phase one. B, the ratio of social time in phase one. C, representative movement track of rats in phase two. D, the ratio of social time in phase two. $^{*}P < 0.05$, compared with VPA group on day 35. $^{\#}P < 0.05$, the same group comparison on day 21 and 35.

3.2 Open-field test

The anxiety behavior of rats was assessed in accordance with their movement time in the central area of the experimental apparatus. The shorter the rats' movement time in the central area was, the severer their anxiety was [25]. The results showed a significant difference between rats in dorsal group after Tuina and those in VPA group ($P < 0.05$), and the anxiety levels of dorsal group showed a reduction. There was no significant difference observed between rats in abdominal and dorsal groups ($P > 0.05$, Figure 3).

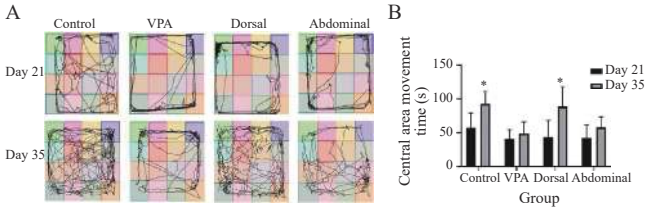

Figure 3 Open-field test on day 21 and 35 ($n = 7$)

A, representative movement track in open-field test. B, the central area movement time. $^{*}P < 0.05$, compared with VPA group on day 35.

3.3 Marble-burying test

The stereotypical behaviors of the experimental rats were assessed based on the number of marbles buried. An increase in the number of marble buried corresponded to a heightened severity of stereotypical behaviors [26]. The results showed that the stereotypical behaviors of rats in dorsal and abdominal groups were reduced in varying degrees after the intervention compared with VPA group ($P < 0.01$ in dorsal group and $P < 0.05$ in abdominal group), with a statistically significant difference between abdominal and dorsal groups ($P < 0.05$, Figure 4).

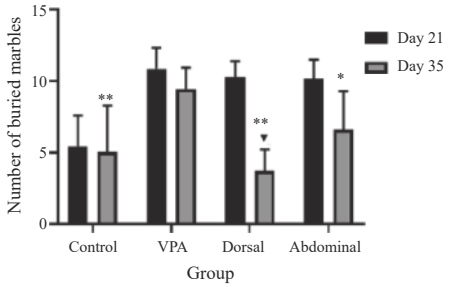

Figure 4 Number of buried marbles in marble-burying test on day 21 and 35 ($n = 7$)

$^{*}P < 0.05$ and $^{**}P < 0.01$, compared with VPA group on day 35. $^{\blacktriangledown}P < 0.05$, compared with abdominal group on day 35.

3.4 Gut microflora diversity

The species detected in the 28 intestinal content samples using QIIME2 (2019.4) belonged to 22 phyla, 42 classes, 74 orders, 132 families, and 259 genera. The α-diversity analysis compared the Chao1 and Shannon indexes of rats in control, VPA, dorsal, and abdominal groups. The Chao1 index mainly reflects the number of species, namely the richness. And the Shannon index reflects the diversity of species, which can be used to measure the uniformity and distribution of different species in the sample. The results showed that the Chao1 index of rats in dorsal and abdominal groups significantly increased when compared with VPA group ($P < 0.05$), indicating that Tuina increased the richness of gut microflora in ASD rats in dorsal and abdominal groups. However, there was no significant difference observed in the alteration of microflora richness between the two groups ($P > 0.05$, Figure 5A). Compared with VPA group, the Shannon index of dorsal group was markedly increased ($P < 0.05$), and the difference of Shannon index between abdominal and VPA groups was more significant ($P < 0.01$, Figure 5B). The results indicated that Tuina in dorsal and abdominal areas increased the diversity of gut microflora of ASD rats, with more prominent effects observed in abdominal group. The β-diversity analysis employed PcoA based on the weighted UniFrac distance. Statistical tests were performed with the use of permutational multivariate analysis of variance (PERMANOVA). The results showed a significant difference in the species of the gut microflora between dorsal and abdominal groups after Tuina when compared with VPA group ($P < 0.01$). However, no significant difference was found in the gut microflora composition between dorsal and abdominal groups ($P > 0.05$, Figure 5C and Table 1).

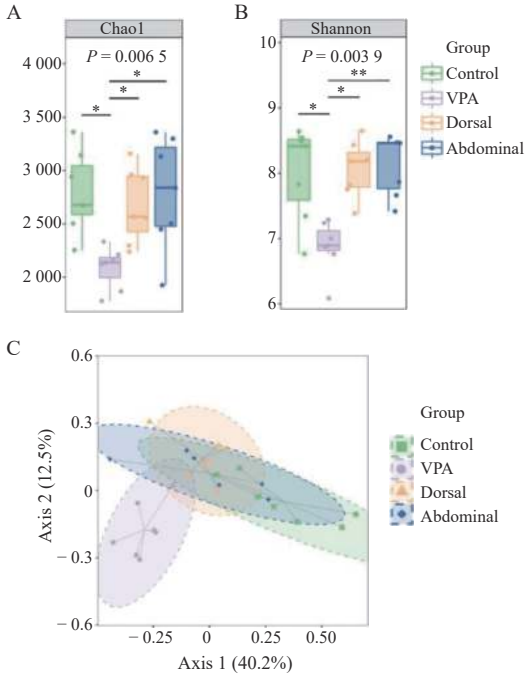

Figure 5 α-Diversity and β-diversity analyses on day 35 ($n = 7$)
A, Chao1 index of gut microflora. B, Shannon index of gut microflora. C, β-diversity analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with VPA group.

Table 1 β-Diversity analysis of gut microflora ($n = 7$)

Group		PERMANOVA	
		Pseudo F	*P* value
Dorsal	Abdominal	1.173	0.253
	VPA	5.943	0.001
	Control	3.371	0.001
Abdominal	VPA	5.538	0.001
	Control	3.257	0.001
VPA	Control	5.327	0.002

3.5 Gut microflora differences

At the phylum level, *Firmicutes* and *Bacteroidetes* were the predominant species in four groups, albeit in varying proportions. The relative abundances of *Firmicutes* of rats in control, VPA, dorsal, and abdominal groups were 55.67%, 80.28%, 71.68%, and 67.93%, respectively. Meanwhile, the relative abundances of *Bacteroidetes* in rats in control, VPA, dorsal, and abdominal groups were 40.65%, 15.75%, 22.37%, and 26.56%, respectively. The results showed that, compared with VPA group, the relative abundance of *Firmicutes*in abdominal group after Tuina showed a significant trend of decrease ($P < 0.05$), and the relative abundances of *Bacteroidetes* in dorsal and abdominal groups was higher than VPA group but had no statistical significance ($P > 0.05$), while the relative abundance of *Firmicutes* and *Bacteroidetes* in dorsalgroup was not significantly different from abdominal group ($P > 0.05$, Figure 6A and Table 2).

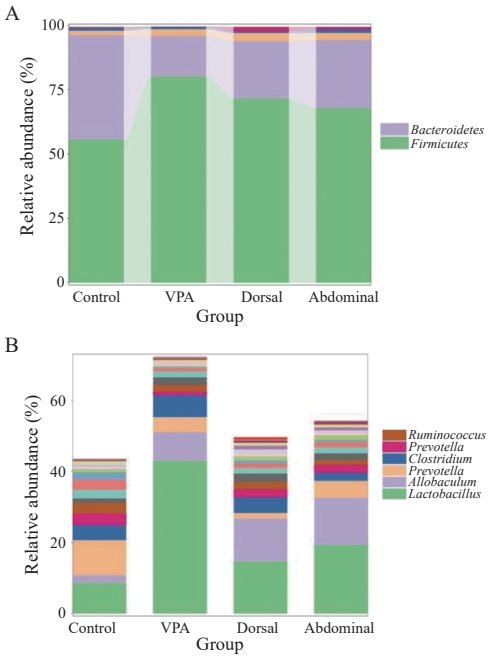

Figure 6 Relative abundance species ($n = 7$)
A, the phylum level. B, the genus level.

At the genus level, *Lactobacillus* were enriched in VPA group, compared with VPA group, the relative abundance of *Lactobacillus* in both dorsal and abdominal groups decreased after intervention ($P < 0.05$). Compared with control group, the relative abundance of *Lactobacillus* in dorsal group was not significantly different ($P > 0.05$); and there was no significant differences between dorsal and abdominal groups ($P > 0.05$), suggesting that Tuina could reduce the content of *Lactobacillus* in the intestinal flora of ASD rats (Figure 6B and Table 2).

Table 2 Gut microflora differences in phylum and denus level [$\bar{x} \pm s$ (%), $n = 7$]

Group	Phylum		Genus	
	Firmicutes	*Bacteroidetes*	*Lactobacillus*	*Allobaculum*
Control	55.67 ± 6.94*	40.65 ± 7.00*	8.62 ± 2.52*	2.23 0.76
VPA	80.28 ± 1.61$^{▲}$	15.75 ± 1.18$^{▲}$	43.00 ± 3.50$^{▲}$	8.05 1.33
Dorsal	71.68 ± 2.98$^{▲}$	22.37 ± 2.53$^{▲}$	14.67 ± 2.12*	12.07 2.40$^{▲}$
Abdominal	67.93 ± 2.92$^{*▲}$	26.56 ± 3.04$^{▲}$	19.40 ± 2.57$^{*▲}$	13.23 4.00$^{▲}$
P value	0.003	0.002	< 0.001	0.018

$^{*}P < 0.05$, compared with VPA group. $^{▲}P < 0.05$, compared with control group.

3.6 Differential species and marker species

Species with significantly different relative abundance at each taxonomic level in VPA, dorsal, and abdominal groups were identified through linear discriminant analysis effect size (LEfSe) analysis [$P < 0.05$ and the linear discriminant analysis (LDA) score > 2]. LDA score was employed to evaluate the influences of species with significant differences, with larger score, greater difference, indicating higher abundance in that particular group than in other groups. The default LDA score threshold was set to 2, and species with scores greater than 2 were displayed. The results showed that at the genus level, *Blautia*, *Phascolarctobacterium*, *p_75_a5*, *Coprobacillus*, and *02d06* were significantly elevated in dorsal group ($P < 0.05$), and *Prevotella*, *Helicobacter*, *Parabacteroides*, and *rc4_4* were significantly elevated in abdominal group ($P < 0.05$, Figure 7). According to the logarithmic order of LDA scores, the most significant change in gut microbiota in dorsal group was *Blautia*, while that in abdominal group was *Prevotella*, indicating a significant increase in their abundance after Tuina.

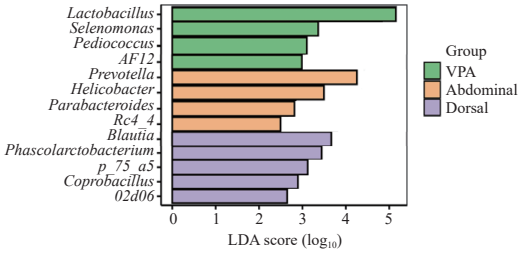

Figure 7 Linear discriminant analysis of gut microflora

4 Discussion

4.1 Relationship between ASD, Gut microflora, and Tuina

4.1.1 Gut microflora and ASD Intestinal microecological balance plays an important role in the development and maturation of newborn's immune and endocrine systems. Disruption of this balance is closely related to the development of various diseases, including ASD [27]. SHARON et al. [4] transplanted the intestinal microflora of ASD children and healthy children into the intestine of germ-free mouse models, and found that mice in the experimental group developed ASD-like behaviors, indicating the importance of intestinal microflora and its metabolites in ASD behaviors [4]. Additionally, a correlation was found between intestinal microecology and the severity of ASD behaviors [28]. Study has reported alterations in the structure of gut microflora in ASD children, noting a reduced diversity in gut microflora compared to their neurotypical counterparts [29]. Moreover, researchers discovered a significant correlation between the diminished diversity and abundance of gut microflora in ASD children and the severity of ASD symptoms [27, 30]. In ASD rat models, alterations in intestinal permeability and gut microflora were observed together with ASD-like behaviors. The restoration of intestinal permeability and gut microflora structure corresponded with improvements in social interactions, anxiety levels, sensorimotor functions, and repetitive stereotypical behaviors [31]. Gut microbiota-based interventions could improve both behavioral and gastrointestinal symptomsin ASD children [32]. In a study conducted by KANG et al. [33], 18 ASD children underwent oral normal intestinal microflora preparation for eight weeks. The results showed that this intervention increased the diversity of intestinal microflora in ASD children, and significantly improved their behavioral and gastrointestinal symptoms. Notably, the positive effects persisted for over eight weeks even after the treatment was discontinued, with minimal adverse events.

4.1.2 Tuina and ASD Tuina has been reported to have effects on ASD symptoms, especially gastrointestinal symptoms [14]. Moreover, there is evidence suggesting Tuina influences the structure of gut microflora in ASD children [34]. Chiropractic is a commonly used Tuina technique, which can increase the colonization of beneficial bacteria in the intestines of children, inhibit the growth of potential pathogenic bacteria, improve the distribution of microbial community structure, and enhance intestinal colonization resistance [35, 36]. Abdominal Tuina can also regulate the structure of intestinal microbiota, increase the number of probiotics, reduce the relative abundance of pathogenic bacteria, enhance intestinal barrier function, and restore intestinal microbiota homeostasis, thereby improving gastrointestinal symptoms and reducing inflammatory response in the human body [15, 37]. Brain-gut peptides, as an important part of the gut-brain axis, are closely related to the gut microbiota. Studies have shown that Tuina can improve the disturbance of serum brain-gut peptides in patients and alleviate related clinical symptoms [38]. Therefore, Tuina could potentially improve ASD symptoms by regulating the gut microflora.

4.2 Effects of Tuina targeting different body parts of ASD patients

The impact of gut microbiota on brain function and behavior through MGBA is implicated in the manifestation of ASD-like symptoms [39, 40]. In addition to the related ASD-like behavior in ASD model rats, the intestinal flora also changed, and when the intestinal flora structure was recovered, the social, anxiety, sensorimotor, repetitive, and stereotyped behaviors also improved [31]. Clinically, it has been found that Tuina has obvious effects on ASD symptoms, especially gastrointestinal symptoms, which can enhance the intestinal barrier function [14], and regulate the intestinal microbial homeostasis [37]. Therefore,

Tuina may improve the core symptoms of ASD by regulating the intestinal microbiota.

4.2.1 Effects on gut microflora At the level of intestinal microorganisms, the proportion of *Firmicutes*/*Bacteroidetes* significantly increased in ASD rat models, which was consistent with previous reports [41, 42]. *Lactobacillus* concentration was also significantly increased in ASD rat models, and decreased in dorsal and abdominal groups after Tuina. *Lactobacillus*, a common probiotic, is able to balance mucosal responses and maintain intestinal microbial homeostasis. However, its impact on regulating abnormal ASD behavior appears to vary, with inconsistent findings of both up-regulation and down-regulation in different studies. This inconsistency underscores the need for further investigation [43]. The gut microflora species composition of rats in dorsal and abdominal groups was significantly altered compared with VPA group, albeit the difference was not significant. The richness and diversity of gut microflora increased in rats in both groups, with abdominal group presenting a more pronounced change in microflora diversity and more uniform species distribution than dorsal group. The DU is connected to the spleen meridian. The Yang-Qi of the DU plays a role in influencing spleen Yang and preserving the homeostasis of intestinal microbial activity [44]. By targeting the abdomen as the site of stimulation, abdominal group demonstrated the ability to enhance intestinal motility and regulate the balance between intestinal activity and microflora ratio [45]. This approach directly contributed to a more pronounced improvement in the structure of gut microflora. Consequently, the species were more evenly distributed in the intestine of the rats in abdominal group after intervention.

Analysis of marker species of rats in dorsal group revealed a significant increase in *Blautia* in the intestines of ASD rats following dorsal Tuina. *Blautia* could increase the expression of tryptophan hydroxylase in the intestines, thus affecting the synthesis and metabolism of 5-hydroxy-tryptamine (5-HT) [46]. Additionally, the abundance of *Blautia* was significantly and positively correlated with intestinal 5-HT levels [47]. 5-HT, a monoamine neurotransmitter, plays a crucial role in regulating central neurotransmission and gut physiological functions. The dysfunction of the 5-HT system is implicated in the development of ASD. It plays an important role in social interactions, stereotypical behaviors, and sensory development [48], which could improve behaviors by regulating physiological functions in animals such as reproduction, mood, aggression, and hormone release. Several studies have identified reduced levels of specific genes responsible for regulating 5-HT synthesis in ASD children, the decrease in 5-HT levels may cause neural network dysfunction [49], consistent with findings from previous studies that have highlighted neural network dysfunction in patients with ASD [50]. Tuina on the dorsal part resulted in a significant improvement in abnormal behaviors, such as stereotypical tendencies, social impairment, and anxiety in ASD rats. This improvement may be related to the increase in *Blautia* in the intestines, potentially activating the synthesis of 5-HT. However, the specific mechanism underlying this improvement needs to be further investigated.

4.2.2 Effects on behaviors In this study, stimulation on the dorsal and abdominal parts improved the behaviors of infant ASD rat models at a varying level. Rats in dorsal group showed significant improvement in social interactions, anxiety levels, and stereotypical behaviors. Conversely, rats in abdominal group showed significant improvement in stereotypical behaviors only, albeit the improvement was less pronounced when compared with dorsal group. In ASD patients, the primary affected area is the brain. Rat models in dorsal group were stimulated in the DU, which ran through the central axis of the body, and were closely connected to the brain in terms of meridian subordination. The activation of the DU facilitates the unblocking of meridians and the regulation of Qi and blood flow, directly contributing to the enhancement of brain functions [51]. The DU is often referred as “the sea of Yang meridians”, playing a crucial role in generating Yang-Qi in the body. It serves as a key component in the production of Yang-Qi [52]. The stimulation of the DU could contribute to the replenishment Yang-Qi, which forms the foundation for normal human activity. And adequate Yang-Qi levels are associated with improvements in behavioral symptoms related to ASD. Therefore, more pronounced improvement in ASD behavioral symptoms was observed through stimulation on the dorsal part, and less prominent results were observed in abdominal group. Tuina may be effective when applied to both dorsal and abdominal parts of ASD patients.

4.2.3 Shortcomings and prospects During the VPA modeling process, pregnant rats were affected by the drug, leading to a high mortality rate and a low success rate in inducing ASD in the offspring. Therefore, future studies should consider increasing the number of pregnant rats injected with VPA to ensure a sufficient sample size of ASD offspring. In this experiment, we used a homemade pushing method stimulator for intervention and specified the stimulation parameters, which standardized the basic research and made it controllable and reproducible. However, the potential adverse effects of machine stimulation on rats need to be further investigated in future experiments.

5 Conclusion

This study demonstrates that Tuina is an effective intervention for ASD, as it improves ASD-related behaviors and modulates the structure of gut microflora in ASD rat models, with more pronounced improvements in ASD

behavior and more significant changes in gut microflora diversity compared with abdominal Tuina.

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Competing interests

The authors declare no conflict of interest.

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推拿不同部位对孤独症谱系障碍模型鼠行为学及肠道菌群的影响

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【摘要】目的 探究不同部位的推拿对丙戊酸（VPA）诱导的孤独症谱系障碍（ASD）模型鼠行为学和肠道菌群的影响。**方法** 将 20 只怀孕 12.5 天的 Sprague Dawley（SD）雌性大鼠随机分为 VPA 模型组 [腹腔注射 VPA（600 mg/kg），$n = 15$] 和生理盐水组（腹腔注射等量生理盐水，$n = 5$）。将注射生理盐水的子代雄鼠作为对照组，将注射 VPA 的子代雄鼠随机分为 VPA 组、背部组和腹部组（$n = 7$）。在出生后第 21 天，通过三箱社交、旷场实验和埋珠实验观察四组大鼠的社交能力、焦虑行为和刻板行为，并开始对背部组和腹部组进行推拿，每天 2 次，持续 14 天。第 35 天，再次进行行为学检测，并取肠道内容物进行物种组成与结构分析、标志物种与差异物种分析。**结果** （1）行为学结果显示，与 VPA 组相比，背部组大鼠社交时间和旷场中心区运动时间显著增加（$P < 0.05$），埋珠数量明显减少（$P < 0.01$），说明其社交、焦虑和刻板行为得到改善；与 VPA 组相比，腹部组大鼠埋珠数量显著减少（$P < 0.05$），刻板行为改善；埋珠实验中，背部组较腹部组埋珠数量更少，刻板行为改善更显著（$P < 0.05$），而三箱社交和旷场实验两组结果无显著差异（$P > 0.05$）。（2）肠道微生物检测结果显示，在多样性分析中，与 VPA 组相比，背部组和腹部组肠道菌群丰富度显著增加（$P < 0.05$），多样性增加（背部组 $P < 0.05$，腹部组 $P < 0.01$）；在差异性分析中，在门水平上，与 VPA 组相比，腹部组厚壁菌门相对丰度呈现较为明显的降低趋势（$P < 0.05$）；在属水平上，与 VPA 组相比，背部组和腹部组的乳杆菌属相对丰度较 VPA 组显著下降（$P < 0.05$）；标志物种分析中，与 VPA 组相比，背部组布劳特氏菌属显著增加（$P < 0.05$）。**结论** 推拿能对 ASD 模型大鼠行为学和肠道菌群结构产生影响；推拿背部对 ASD 模型鼠的社交能力、焦虑行为和刻板行为疗效显著，推拿腹部仅对刻板行为有明显效果；推拿背部与推拿腹部均使 ASD 模型鼠肠道菌群丰富度和多样性增加，其中推拿腹部对肠道微生物多样性的改善更显著，并使物种分布更均匀。

【关键词】推拿；孤独症谱系障碍；动物行为学；肠道菌群；中医；外治法