

## Research Report

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# Fecal and Blood Microbial 16s rRNA Gene Alterations in Chinese Patients with Multiple System Atrophy and Its Subtypes

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### Abstract.

**Background and Objective:** To explore the alterations of microbial 16s ribosomal (rRNA) gene in the feces and blood of Chinese patients with multiple system atrophy (MSA) and its relationships with clinical features.

**Methods:** 40 MSA patients (MSA-P/MSA-C: 23/17) and their healthy spouses were recruited. Fecal and blood microbiota were investigated by high-throughput Illumina Miseq sequencing targeted on the V3-V4 functional region of 16s rRNA gene. The relationships between microbiota and clinical characteristics were analyzed.

**Results:** The abundances of *Lactobacillus*, *Gordonibacter*, *Phascolarctobacterium*, and *Haemophilus* in feces and abundances of *Leucobacter*, and *Bacteroides* in blood were different between MSA patients and healthy controls (HC). Combining the taxa from feces and blood, six genera were identified to be predictive of MSA, achieving an area under the curve (AUC) of 0.853. The abundances of *Phascolarctobacterium* and *Ruminococcus* in feces were lower in MSA-P than those in MSA-C. The abundances of *Blastococcus*, *Bacillus*, and *Acinetobacter* in blood were different between MSA subtypes. These five genera differentiated MSA subtypes with an AUC of 0.898. Functional predictions indicated that gene functions involving biosynthetic metabolism and bacterial secretion systems were significantly different between the MSA and HC. The differential genera were associated with disease duration, anxiety, and autonomic dysfunctions.

**Conclusions:** We confirmed the alterations of microbial 16s rRNA gene in the feces and blood occurs in Chinese patients with MSA. Microbiota dysbiosis was related to MSA clinical manifestations. Elucidating these differences in microbiomes will be helpful to improve our knowledge of the microbiota in the pathogenesis of MSA.

**Keywords:** Multiple system atrophy, 16s rRNA gene, microbiota, blood, feces

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## INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurodegenerative disease characterized by progressive autonomic failure, parkinsonism, cerebellar ataxia, and pyramidal dysfunction [1]. Gastrointestinal symptoms are frequent complaints of patients with MSA due to autonomic nervous system dysfunction. Similar to idiopathic Parkinson's disease (PD), subjects with MSA suffer from multiple gastrointestinal symptoms including constipation [2–4], fecal incontinence [5, 6], delayed gastric emptying [4, 5], slow colonic transit, and abnormal electromyography of the external anal sphincter [4, 6, 7]. The pathological hallmark is the formation of  $\alpha$ -synuclein-containing glial cytoplasmic inclusions (GCIs) in olivopontocerebellar and striatonigral areas [8, 9], and  $\alpha$ -synuclein deposits can also be observed in the enteric nervous system of MSA [10].

Gastrointestinal dysfunction is closely related to gut microbiota dysbiosis. Gut microbiota can regulate both the enteric and central nervous system activities through neural, endocrine and metabolic mechanisms according to the concept of the microbiota-gut-brain axis [11]. Alteration of gut microbiota was associated with a series of neurodegenerative diseases [11, 12], including PD [13–16]. There are two reports of intestinal microflora dysbiosis in MSA patients from different populations [17, 18], such as subjects from Malaysia [17], and from Chicago [18], however, the results of these two studies with small sample sizes were totally different, there is no report in the Chinese patients with MSA so far.

It has long been thought that blood is a sterile environment [19]. However, sequence-based studies have uncovered that blood microbiota were associated with non-communicable disease [20–22]. Microscopic measurement revealed that microbes were in close proximity to the red blood cells (RBCs) of patients with PD [19]. Once the alterations of microbial 16s ribosomal RNA (rRNA) gene in the blood of patients with PD were reported [23], whether the 16s rRNA gene in the blood of MSA patients were changed has not been reported yet.

Our study mainly explored the alterations of microbial 16s rRNA gene in the feces and blood of Chinese patients with MSA and their healthy spouses. We also established a disease diagnosis and differential diagnosis model for MSA in the combination of fecal and blood 16s rRNA genes. The associations between microbiota and clinical variables were also analyzed in detail.

## MATERIALS AND METHODS

### Subjects and clinical evaluation

57 patients with MSA and 54 health controls (HCs) were recruited at first in our study. All subjects were enrolled from the movement disorder clinic at Department of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine during the period from October 2016 to May 2018. Patients were diagnosed as probable MSA according to the second consensus criteria for MSA by senior movement disorder specialists [1]. HCs who had no signs of parkinsonism or potential premotor symptoms were enrolled from the spouses of the patients who lived with patients for at least 20 years to minimize variation caused by different living environments. A total of 40 MSA and 40 HC subjects were included in the final analysis (Fig. 1). Demographic data (age, gender, and BMI (kg/cm<sup>2</sup>)) in all subjects and clinical features in MSA patients including disease duration, levodopa equivalent daily dosage (LEDD), constipation condition, motor and non-motor symptoms were recorded in our study. Exclusion criteria and clinical evaluation scales were seen in the Supplementary Methods. To verify the significantly different genera identified in feces using real-time polymerase chain reaction (PCR), another independent group of 40 probable MSA patients and 40 HCs was recruited. This study protocol was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine. All subjects consented to cooperate

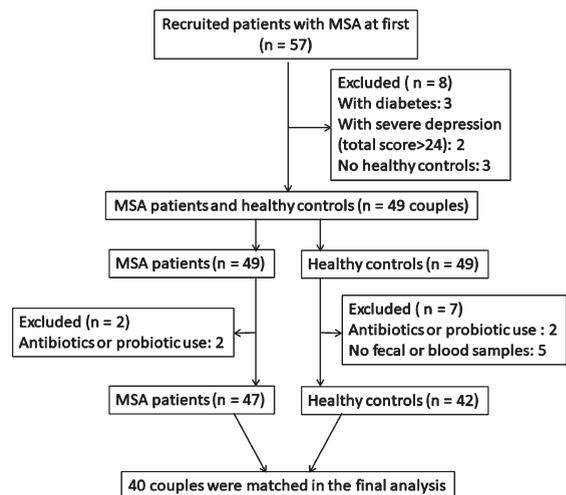


Fig. 1. Recruitment flowchart illustrating the couples based on the exclusion and inclusion criteria.

with this study, the written informed consents were obtained from all subjects in the study as well.

#### *Fecal and blood sample collection and DNA extraction*

Fecal DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) after the sample collection. The blood DNA was extracted from peripheral blood leukocytes using a classical phenol/chloroform extraction method [22]. Regarding potential contamination of the study, fecal and blood DNA extractions were conducted carefully to decrease the risk of contamination by the operating steps. All the DNA extraction were performed by one person within 4 days. Detailed stool and blood collection requirements were seen in the Supplementary Methods.

#### *Analysis of microbial DNA*

After extraction of total 16s rRNA gene from both the fecal and blood samples, PCR amplification was carried out for high-throughput Illumina Miseq sequencing targeted on the V3-V4 functional region of the 16s rRNA gene. Detailed amplification, sequencing, and analysis processes were described in the Supplementary Methods. After quality control and merge, an average of 40000 reads per sample were selected, and numbers of the optimized sequences were shown in Supplementary Table 1.

#### *Genus-specific quantification by Real-Time PCR*

The 16S rRNA gene copies of each sample was evaluated by real-time PCR using universal forward and reverse primers, EUBF: 50-GCTCGTGTCGTGAGATGTT-30 and EUBR: 50-TGTAGCCCAGGT CATAAGG-30. The genus-specific primer sequences used in this study are listed in Supplementary Table 2.

#### *Statistical analysis*

Mothur and R version software were used for the statistical analysis. Continuous data were presented as mean and standard deviation (mean  $\pm$  SD) and compared by Wilcoxon rank-sum test. Categorical variables were presented as numbers/percentage and compared between groups using chi-squared ( $\chi^2$ ) test. For taxa with a prevalence  $\geq 10\%$ , differences in microbial abundances between patients and controls were analyzed using Wilcoxon rank-sum test at the

phylum, class, order, family, genus, and species levels. For multiple comparisons of bacterial analysis, the false discovery rate (FDR) was calculated. We chose the genus level for further analysis (Supplementary Methods).

## RESULTS

### *Demographics and clinical data*

Demographic and clinical characteristics of patients with MSA and HCs are presented in Table 1. No differences were observed in age, gender, BMI, history of smoke, alcohol, tea, or yogurt consumption between MSA and HC groups ( $p > 0.05$ ). MSA patients had higher percentage of constipation than HC group ( $p < 0.001$ ). The severity of constipation in MSA patients was much more obvious than in HC subjects assessed by Wexner Scale ( $p < 0.001$ ). MSA patients had mean disease duration of  $3.93 \pm 1.91$  years, LEDD of  $563.78 \pm 405.64$  mg/d, UMSARS-II score of  $21.3 \pm 9.4$ , MMSE score of  $26.0 \pm 3.1$ , and SCOPA-AUT score of  $22.2 \pm 9.8$ .

### *Alterations of microbiota*

#### *Comparisons of microbiota between MSA and HC group*

A total of 26 phyla, 45 classes, 68 orders, 108 families, and 222 genera were identified in gut microbiota in MSA and HC groups. No significant difference was observed in  $\alpha$ -diversity (Fig. 2A).  $\beta$ -diversity based on Bray-Curtis did not show any significant difference ( $p > 0.05$ , FDR  $p > 0.05$ , data not shown). Also, there was no difference in the weighted Unifrac-based  $\beta$ -diversity ( $p = 0.243$ ; FDR  $p = 0.441$ ). However, the unweighted Unifrac-based  $\beta$ -diversity revealed a significant difference between MSA and HC groups ( $p = 0.001$ ; FDR  $p = 0.021$ ) (Fig. 2B). This indicated the composition of fecal microbiota in MSA patients was significantly different from that in HC group. Here we only paid attention to the genus level between these two groups. We used LEfSe analysis to investigate the specific changes of the microbial abundance. The mean relative abundant genera of *Scardovia*, *Lactobacillus*, *Gordonibacter*, and *Phascolarctobacterium* were higher in MSA group, while the mean relative abundant genus of *Haemophilus* was lower in MSA group than that in HC group (Fig. 3A&B, LDA score ( $\log_{10}$ )  $> 2$ ). To control the effects of confounding factors (age, gender, BMI, constipation, LEDD, depression, and anxiety) on the

Table 1  
Demographic data and clinical characteristics in all subjects

	MSA (n = 40)	MSA-P (n = 23)	MSA-C (n = 17)	HC (n = 40)	p value <sup>c</sup>	p value <sup>d</sup>
Age(y) <sup>a</sup>	60.98 ± 6.7	62.48 ± 5.48	58.94 ± 7.83	60.88 ± 7.0	1.000	0.157
Male% (male/female) <sup>b</sup>	57.5% (23/17)	52.2% (12/11)	64.7% (11/6)	42.5% (17/23)	0.180	0.525
BMI (kg/cm <sup>2</sup> ) <sup>a</sup>	23.93 ± 3.25	24.10 ± 3.10	23.70 ± 3.53	23.78 ± 2.80	1.000	0.529
Wexner <sup>a</sup>	8.40 ± 5.30	8.78 ± 5.41	7.88 ± 5.27	1.35 ± 2.17	<0.001	0.621
Constipation% (±) <sup>b</sup>	75% (30/10)	69.6% (16/7)	82.3(14/3)	12.5% (5/35)	<0.001	0.471
HAMD-17 <sup>a</sup>	11.63 ± 4.72	12.43 ± 5.09	10.53 ± 4.05	0.85 ± 1.61	<0.001	0.336
HAMA <sup>a</sup>	12.18 ± 5.12	12.48 ± 4.80	11.76 ± 5.65	0.73 ± 1.59	<0.001	0.681
Smoke (±) <sup>b</sup>	10% (4/36)	4.3% (1/22)	17.6% (3/14)	20% (8/32)	0.348	0.294 <sup>e</sup>
Alcohol (±) <sup>b</sup>	17.5% (7/33)	8.7% (2/21)	29.4% (5/12)	15% (6/34)	1.000	0.113 <sup>e</sup>
Tea (±) <sup>b</sup>	17.5% (7/33)	13.0% (3/20)	23.5% (4/13)	12.5 (5/35)	0.754	0.432 <sup>e</sup>
Yogurt (±) <sup>b</sup>	25% (10/30)	21.7% (5/18)	29.4% (5/12)	32.5% (13/27)	0.621	0.717 <sup>e</sup>
Disease duration (y) <sup>a</sup>	3.93 ± 1.91	4.52 ± 1.68	3.12 ± 1.97	/	/	0.014
LEDD (mg/d) <sup>a</sup>	563.78 ± 405.64	802.22 ± 313.554	241.18 ± 271.273	/	/	<0.001
UMSARS-II <sup>a</sup>	21.3 ± 9.4	25.87 ± 8.66	15.18 ± 6.57	/	/	<0.001
MMSE <sup>a</sup>	26.0 ± 3.1	25.57 ± 3.30	26.59 ± 2.83	/	/	0.423
SCOPA-AUT <sup>a</sup>	22.2 ± 9.8	22.52 ± 10.35	21.65 ± 9.30	/	/	0.956

<sup>a</sup>Data were shown as mean ± SD, compared by Wilcoxon test. <sup>b</sup>Data were shown as number %, compared by  $\chi^2$  test. <sup>c</sup>Compared between MSA and HC. <sup>d</sup>Compared between MSA-P vs MSA-C. <sup>e</sup>Fisher test. MSA, Multiple system atrophy; MSA-P, multiple system atrophy with predominately parkinsonism; MSA-C, multiple system atrophy with predominately cerebellar ataxia; HC, Health controls; BMI, Body Mass Index; HAMD-17, 17-item Hamilton Rating Scale for Depression; HAMA, Hamilton Anxiety Scale; LEDD, levodopa equivalent daily dosage; UMSARS-II, the Unified Multiple System Atrophy Rating Scale-II; MMSE, Mini-Mental State Examination; SCOPA-AUT, The Scale for Outcomes in PD autonomic dysfunction.

abundance differences, GLMs were applied to model the differences of bacterial abundances (Supplementary Table 3). Finally, the increased abundances of *Lactobacillus*, *Gordonibacter*, and *Phascolarctobacterium*, and decreased abundances of *Haemophilus* were significantly related to MSA diagnosis.

Blood microbiota was also detected in MSA and HC group. A total of 24 phyla, 31 classes, 70 orders, 92 families, and 156 genera were identified in blood microbiota. No significant difference was observed in  $\alpha$ -diversity (Supplementary Figure 1A).  $\beta$ -diversity based on Bray-Curtis did not show significant difference ( $p > 0.05$ , FDR  $p > 0.05$ , data not shown). However, the unweighted and weighted Unifrac-based  $\beta$ -diversity revealed significant differences between MSA and HC groups (unweighted  $p = 0.018$  (FDR,  $p = 0.029$ ); weighted  $p = 0.047$  (FDR,  $p = 0.034$ )) (Supplementary Figure 1B). With LefSe analysis, the mean relative abundant genus of *Bacteroides* was higher in MSA group, while the mean relative abundance of *Leucobacter* was lower in MSA group (Fig. 3C, LDA score (log<sub>10</sub>) > 2). Finally, the increased abundance of *Bacteroides* and decreased abundance of *Leucobacter* were significantly related to MSA diagnosis based on GLMs adjusting for age, gender, BMI, constipation, LEDD, depression, and anxiety (Supplementary Table 3).

To evaluate the diagnostic status for MSA based on specifically differential bacteria by LefSe analysis, receiver operating characteristic curve (ROC) was used to build a predictive model. The combination of 4 differential taxa in feces (*Lactobacillus*, *Gordonibacter*, *Phascolarctobacterium*, and *Haemophilus*) differentiated MSA patients from controls with a modest accuracy (area under the curve [AUC] 0.798, 95%CI 0.702–0.894,  $p < 0.001$ ), yielding a sensitivity of 90.0% and a specificity of 62.5% in the ROC curve (Fig. 3D). However, the combination of *Leucobacter* and *Bacteroides* in blood differentiated MSA patients from controls with an AUC of 0.748 (95%CI 0.640–0.855, sensitivity 60.0% and specificity 85.0%,  $p < 0.001$ ) in the ROC curve (Fig. 3D). Combining the different taxa from the feces and blood, 6 genera were identified to be predictive of MSA, achieving an AUC of 0.853 (95%CI 0.771–0.936, sensitivity 62.5% and specificity 100%,  $p < 0.001$ ) (Fig. 3D).

#### Comparisons of microbiota between MSA subtypes

We also used LefSe analysis to explore the different microbiota between MSA subtypes in order to discriminate these two groups. We first compared the MSA subtypes with their respective HCs to eliminate the environmental factors that could

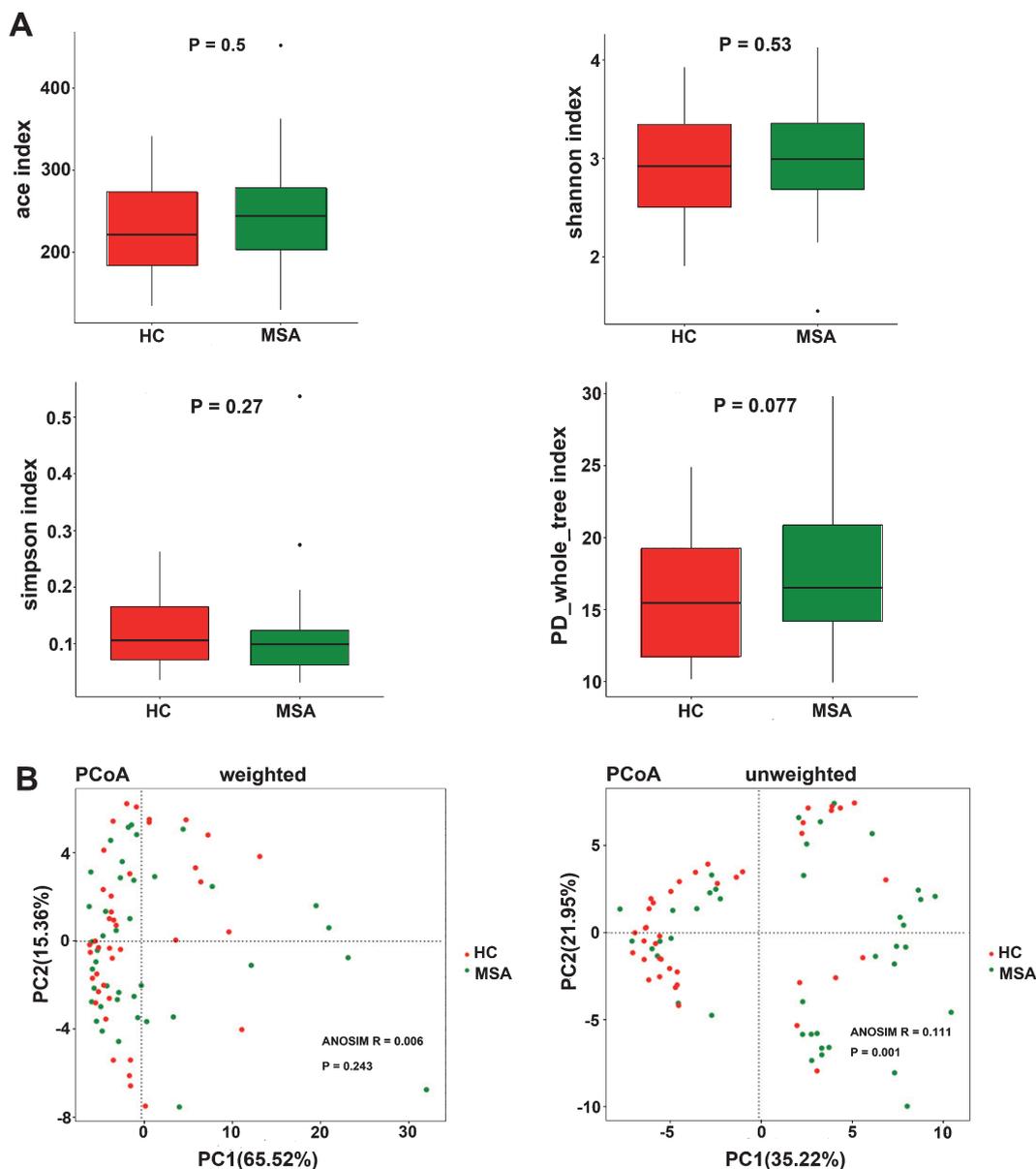


Fig. 2. The  $\alpha$ -diversity and  $\beta$ -diversity of gut microbiota in MSA and HC groups. A) Ace, chao, Shannon, Simpson and PD whole tree indexes were described in box plots and compared by Wilcoxon test. Each box plot represented the median, interquartile range, minimum, and maximum values. B) PCoA depicted the differences of  $\beta$ -diversity based on unweighted and weighted UniFrac dissimilarity matrix. ANOSIM was used to compare the differences of  $\beta$ -diversity between the two groups. The axes represented the principal coordinate component that interprets the data variance. MSA, multiple system atrophy (green); HC, healthy control (red); PCoA, principal coordinate analysis; ANOSIM, analyses of similarity; PC, principal coordinate.

interrupt the differential bacteria. In fecal samples, the mean relative abundant genera of *Phascolarctobacterium* and *Ruminococcus* were lower in MSA-P group than those in MSA-C group (Fig. 3E, Supplementary Table 4), these bacteria were significantly different in the comparison between MSA-P and their corresponding HCs, as well as the comparison

between MSA-C and their matched HC (Supplementary Figure 2A&2B). ROC curve revealed that these two genera together differentiated MSA-C patients from MSA-P patients with an AUC of 0.844 (95%CI 0.722–0.966, sensitivity 82.4% and specificity 78.3%,  $p < 0.001$ ) (Fig. 3G). In blood samples, the mean relative abundance of *Blastococcus* and

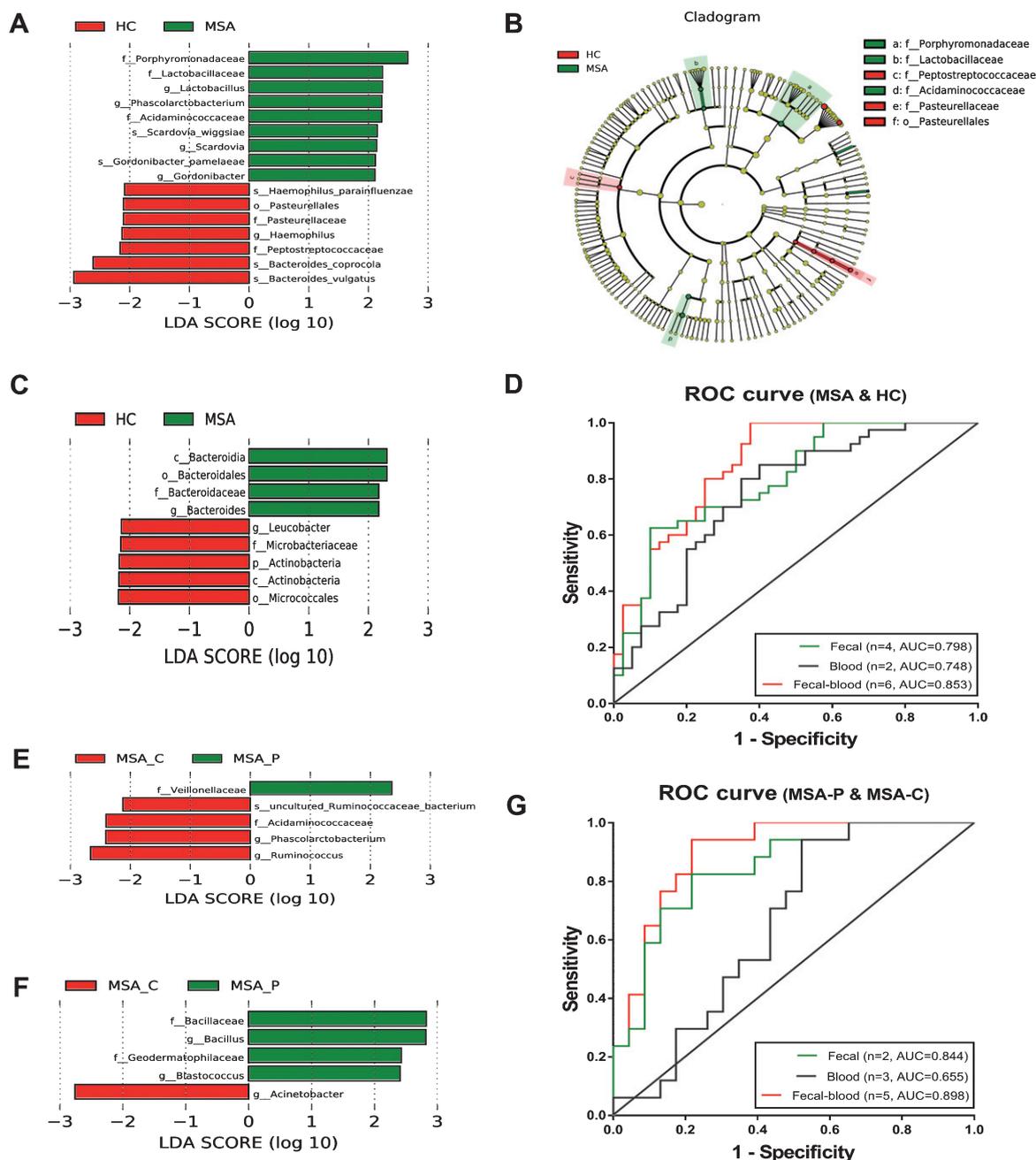


Fig. 3. Taxonomic differences of fecal and blood microbiota. A) Comparisons of gut microbiota between MSA and HC groups. The most differentially abundant taxa in feces between MSA and HC groups were identified through the LDA scores which was generated from LefSe analysis. The LDA scores ( $\log_{10}$ ) $>2$  and  $p < 0.05$  were listed. B) The enriched taxa in MSA and HC groups were listed in Cladogram. The central point represents the highest classified level, and each ring represents the next lower taxonomic level. The diameter of each circle represents the relative abundance of the taxon. C) Comparisons of blood microbiota between MSA and HC group. D) ROC curves were built to evaluate the diagnostic value for MSA based on the specifically differential bacteria by LefSe analysis. With six genera both in feces and blood, MSA could be separated from HCs with an AUC of 0.853. E) Comparisons of gut microbiota between MSA subtypes. F) Comparisons of blood microbiota between MSA subtypes. G) ROC curves were used for the differential diagnosis of MSA subtypes. With five genera both in feces and blood, MSA-P could be separated from MSA-C with an AUC of 0.898. p, phylum; c, class; o, order; f, family; g, genus; s, species; MSA, multiple system atrophy (green); HC, healthy control (red); MSA-P, multiple system atrophy with predominately parkinsonism (green); MSA-C, multiple system atrophy with predominately cerebellar ataxia (red); LDA, linear discriminant analysis; ROC, receiver operating characteristic curve; AUC, area under the curve.

*Bacillus* was increased in MSA-P patients, while *Acinetobacter* was decreased in MSA-P patients compared with MSA-C patients (Fig. 3F, Supplementary Table 4). However, the predictive model could not discriminate well between the MSA subtypes based on differential genera in blood ([AUC] 0.655, 95%CI 0.485–0.825,  $p=0.098$ ) (Fig. 3G). Combining the different taxa from the feces and blood, 5 genera differentiated MSA-C patients from MSA-P patients, achieving an AUC of 0.898 (95%CI 0.801–0.994, sensitivity 94.1% and specificity 78.3%,  $p<0.001$ ) (Fig. 3G).

#### Verification of different genera in feces by Real-Time PCR

Particularly, we designed the genus-specific primers and conducted real-time PCR to verify the changes of these genera based on the LEfSe in feces, the increased abundances of *Phascolarctobacterium*, *Lactobacillus*, *Gordonibacter*, and the decreased abundances of *Haemophilus* were identified in MSA patients using real-time PCR ( $p=0.028$ , 0.001, 0.040, and 0.021, Supplementary Figure 2C). Compared with MSA-C patients, the decreased abundances of *Phascolarctobacterium* and *Ruminococcus* were also identified in MSA-P patients ( $p=0.036$  and 0.018, Supplementary Figure 2E). Furthermore, we verified these genera using real-time PCR in the fecal samples of another independent group of 40 MSA patients and 40 HCs (Supplementary Table 5), the increased *Phascolarctobacterium* ( $p=0.020$ ) and *Gordonibacter* ( $p=0.014$ ) were identified in MSA patients (Supplementary Figure 2D). There was an upward trend in the abundance of *Lactobacillus* ( $p=0.053$ ) and a downward trend in the abundance of *Haemophilus* ( $p=0.751$ ) in MSA patients (Supplementary Figure 2D). Compared with MSA-C patients, the decreased *Phascolarctobacterium* and *Ruminococcus* were also identified in MSA-P patients ( $p=0.039$  and 0.011, Supplementary Figure 2F).

#### Functional prediction of fecal and blood microbiota

In order to predict the functional alterations of the microbes in feces and blood, PICRUSt was applied to estimate the KO abundances using the 16s rRNA gene sequencing data in MSA patients and HCs. Significant difference was identified in 6 KEGG pathways (Level 3) in fecal microbiota. The KEGG pathways involved in synthesis and degradation of

ketone bodies, taurine and hypotaurine metabolism, and bacterial secretion system had higher abundances in MSA patients than that in HCs ( $p<0.05$ ) (Fig. 4A). Conversely, the KEGG pathways including restriction enzyme, linoleic acid metabolism, and RNA transport were less abundant in MSA patients relative to HCs ( $p<0.05$ ) (Fig. 4A).

Significant different pathways were identified both in the level 2 and 3 KEGG pathways in blood microbiota. The level 2 KEGG pathway related to immune system was increased in the blood of MSA patients ( $p<0.05$ ) (Fig. 4B). The level 3 KEGG pathways related to RNA transport, prostate cancer, beta lactam resistance, tetracycline biosynthesis, progesterone mediated oocyte maturation, antigen processing and presentation, NOD like receptor signaling pathway, and restriction enzyme were higher in MSA patients, whereas riboflavin metabolism was lower in MSA patients compared with HCs ( $p<0.05$ ) (Fig. 4C). However, no significantly different KEGG pathways were detected either in feces or blood at an FDR of 5%.

#### Associations between clinical features and microbiota in feces and blood

The associations between altered gut microbes (genus level) and clinical features are listed in Fig. 4D. Gut microbes were weakly associated with clinical symptoms. *Haemophilus* was negatively correlated with disease duration ( $r=-0.399$ ,  $p=0.010$ ), USMARS-II scores ( $r=-0.395$ ,  $p=0.012$ ), HAMD-17 scores ( $r=-0.387$ ,  $p=0.014$ ), and HAMA scores ( $r=-0.346$ ,  $p=0.028$ ). HAMA scores were also positively associated with *Lactobacillus* ( $r=0.386$ ,  $p=0.013$ ) and *Phascolarctobacterium* ( $r=0.333$ ,  $p=0.035$ ). In addition, SCOPA-AUT scores were positively related with *Gordonibacter* ( $r=0.195$ ,  $p=0.011$ ). GLM method was used to adjust the potential confounders (age, gender, BMI, LEDD, disease duration) (Supplementary Table 6). *Haemophilus* was negatively correlated with disease duration ( $p<0.001$ ), HAMA scores were only associated with *Phascolarctobacterium* ( $p=0.002$ ), SCOPA-AUT scores were still positively related with *Gordonibacter* ( $p<0.001$ ).

As for the associations of clinical parameters with blood microbiota (Fig. 4E), we also observed significant positive correlations between *Citricoccus* and clinical features including disease duration ( $r=0.418$ ,  $p=0.007$ ) and UMSARS-II scores ( $r=0.480$ ,  $p=0.001$ ). SCOPA-AUT scores were

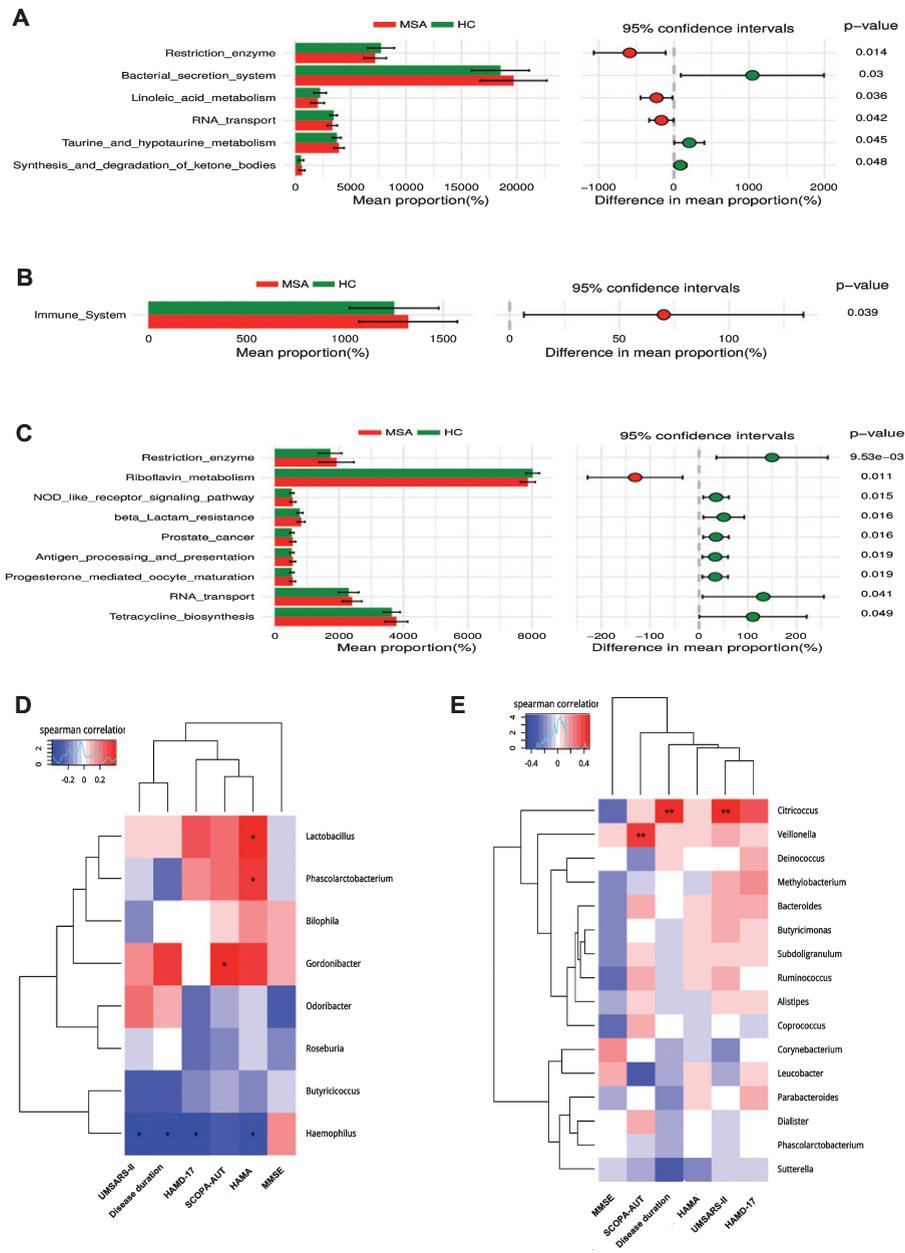


Fig. 4. Functional predictions of the microbiota and its associations between clinical features. Functional predictions of the microbiota in MSA and HC groups were listed in (A), (B), and (C). In feces, significant differences of KEGG pathways at level 3 were identified by STAMP software (A). In blood, significant KEGG pathways at level 2 (B) and 3 (C) were identified. Associations between clinical features and microbiota in feces (D) and blood (E) were listed in the heat maps using Spearman's rank-correlation analysis. The intensity of the color represented the "r" value (negative score, blue; positive score, red). \* $p < 0.05$ , \*\* $p < 0.001$ . KEGG, Kyoto Encyclopedia of Genes and Genomes; UMSARS-II, The Unified Multiple System Atrophy Rating Scale-II; HAMD-17, 17-item Hamilton Rating Scale for Depression; HAMA, Hamilton Anxiety Scale; MMSE, Mini-Mental State Examination; SCOPA-AUT, The Scale for Outcomes in PD autonomic dysfunction.

also positively associated with *Veillonella* ( $r = 0.408$ ,  $p = 0.008$ ). After adjusting for confounders by GLM (Supplementary Table 7), *Citricoccus* was

only related with disease duration ( $p < 0.001$ ), and *Veillonella* was related with SCOPA-AUT scores ( $p = 0.030$ ).

## DISCUSSION

Our study confirmed the alterations of microbiome in patients with MSA and its relationships with clinical features. This is the first report on the intestinal microflora changes in the Chinese MSA population. We also first reported the alterations of the microbiota in the blood and microbial dysbiosis between different MSA subtypes in the world. The predictive model of differential bacterial taxa for MSA in the combination of fecal and blood 16s rRNA genes was first proposed as well. Furthermore, the recruitment of healthy spouses as controls is an important advantage that greatly reduced the impact of different environments and diets on the results. Each couple included in our study have lived together in a family for at least 20 years, so that they share more common microbiota than individuals from different households [24].

Previous studies addressing differences in gut microbiota between MSA patients and HCs were not consistent with our results [17, 18, 25]. A recent study from Malaysia found a reduction in *Paraprecotella* and an increase in *Bacteroides* at the taxonomic level of genus [17]. Another study from Chicago population found a decrease in the abundance of anti-inflammatory bacteria such as *Ruminococcus*, *Roseburia*, *Coprococcus*, and *Faecalibacterium* [18]. It was suggested that race, geographical location, dietary habits, climatic conditions, host genetics, age, and sequencing technologies from different populations might influence the results [26]. As for the different alterations of microbiome between different MSA subtypes, the abundances of *Phascolarctobacterium* and *Ruminococcus* in MSA-P group were lower than that in MSA-C group. A recent study indicated decreased *Ruminococcus* in fecal samples of PD subjects which was consistent with our results in MSA-P group [27]. Particularly, those gut microbial differences between MSA and HCs, including those differences between MSA-P and MSA-C were consistently confirmed in the same fecal samples using real-time PCR.

In our study, MSA differed from HCs both in the fecal and blood microbes, however, the results in the feces and blood showed totally different patterns. It was consistent with our previous findings that the blood and fecal microbial changes in PD were different [23, 28], indicating that blood bacteria may not be derived from the intestine through bacterial translocation directly, which has been proposed in the previous studies [29, 30]. As for the source of the microflora in the blood, Koren suggested that blood

bacteria might be translocated from oral cavity in patients with atherosclerosis [31]. The oral and nasal microbiota changes in PD patients were also reported previously [32], however, the blood microbiota in this study was not detected. Thus, the microbial investigations should not be limited to the intestines, but should be checked in the blood and oral cavity as well in the future. Although the team has not yet been able to fully eliminate the contamination with the help of microbiologists in the operational steps, the discovery in blood will provide more evidence for our understanding of the relationship between microbiota and MSA.

The content of microbial 16s rRNA gene in the blood is much lower than that in the intestinal flora, so the value of blood predictive model for MSA alone was not enough. Thus, we proposed a combined diagnostic model with fecal and blood taxa, a higher AUC was achieved to predict MSA and to differentiate MSA subtypes, which can provide us a new method for the diagnosis of MSA.

The functional predictions inferred significantly different abundances of KEGG pathways both in blood and fecal samples, especially pathways involved in biosynthetic metabolism. Our study also demonstrated the increased abundance of bacterial secretion system pathway in patients with MSA, which was consistent with the functional analysis in PD patients [15]. The activation of bacterial secretion system can promote the formation of bacterial products including inflammatory proteins [33]. However, the PICRUSt through 16s rRNA gene sequencing has its limitations compared with metagenomic analyses, as the functional alterations of the microbes cannot be predicted comprehensively by 16s rRNA gene sequencing. Further study using metagenomic sequencing to study the functional alterations of microbes in MSA patients is necessary.

As for the influence of gut microbiota on clinical symptoms, no analysis in patients with MSA has been performed so far. The increase of *Lactobacillus* and *Phascolarctobacterium* in the feces were weakly associated with anxiety in our results. The relationship between gut microflora and emotional disorders such as anxiety and depression was also reported before [34, 35]. The gram-positive bacteria such as *Lactobacillus* can metabolize glutamate to produce gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the brain. Disorders in the GABA pathway may cause anxiety, depression, and cognitive impairment [36]. In addition, it has also been reported that the increased

abundance of *Lactobacillus* was associated with the decreased level of the gut hormone ghrelin [37], which was seen in patients with PD [38]. In the analysis of MSA blood samples, *Citricoccus* was found to be related with disease duration, and *Veillonella* was related with autonomic dysfunction. No previous study has directly indicated an association between autonomic dysfunction and microbiota.

There are several limitations in our study. First, this study is a cross-sectional study. First of all, the results can not indicate the causal relationship between microbiota and MSA, further study might focus on whether altered microbiota could induce MSA. Secondly, there are many factors that could affect the gut microbiota. Although we chose the spouses as controls to minimize the differences in the dietary habit and living environment, and controlled many potential confounders such as age, gender, BMI, constipation, disease severity, depression and anxiety, the confounding factors could not be completely eliminated. In addition, the microflora of different geographical environments and ethnic groups were different at certain degree, which limits the generalizability of this conclusion. Furthermore, the analysis between MSA and HC is not enough to be accepted as a biomarker, further study should focus on the effect of intestinal microflora on PD and other Parkinson-plus syndrome.

### Conclusions

Overall, we confirmed that the gut and blood microbial alterations existed in Chinese patients with MSA. Additionally, the fecal and blood microbiota was related to clinical features such as disease duration, anxiety, and autonomic dysfunction. KEGG pathways involved in biosynthetic metabolism and bacterial secretion systems were significantly different between the MSA and HC groups. Elucidating these differences in microbiomes will be helpful to improve our knowledge of the microbiota in the pathogenesis of MSA.

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### CONFLICT OF INTEREST

The authors have no conflict of interest to report.

### SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JPD-191612>.

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