

# Gut microbiome and serum metabolome alterations in isolated dystonia

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## Research

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# Abstract

## Background

Dystonia is a complex neurological movement disorder characterised by involuntary muscle contractions. The relationship between the gut microbiota and isolated dystonia remains poorly explored.

## Methods

We collected faeces and blood samples to study the microbiome and the serum metabolome from a cohort of 57 drug-naïve isolated dystonia patients and 27 age- and environment-matched healthy individuals. We first sequenced the V4 regions of the 16S rDNA gene from all faeces samples. Further, we performed metagenomic sequencing of gut microbiome and non-targeted metabolomics profiling of serum from dystonia patients with significant dysbiosis.

## Results

Gut microbial  $\beta$ -diversity was significantly different, with a more heterogeneous community structure among dystonia individuals than healthy controls, while no difference in  $\alpha$ -diversity was found. Gut microbiota in dystonia patients was enriched with *Blautia obeum*, *Dorea longicatena* and *Eubacterium hallii*, but depleted with *Bacteroides vulgatus* and *Bacteroides plebeius*. Metagenomic sequencing revealed that genes related to the citrate cycle, vitamin B6 and glycan metabolism were less abundant in dystonia, while genes linked to purine and tryptophan biosynthesis were more abundant. Serum metabolome analysis revealed altered levels of tyrosine and glutamate. The integrative analysis of the gut microbiome and serum metabolomics identified dystonia-associated gut microbial species linked to changes in serum metabolites, reflecting the effect of the gut microbiome on metabolic activity in isolated dystonia.

## Conclusion

This study is the first to reveal gut microbial dysbiosis in dystonia patients. Our findings identified previously unknown links between intestinal microbiota alterations, circulating amino acids and dystonia, providing new insight into the pathogenesis of isolated dystonia.

## Research In Context

### Evidence before this study

Dystonia is a complex neurological movement disorder and its pathogenesis has not been fully elucidated. Emerging evidence has implicated the gut microbiome as an important player in many

neurological diseases. One case was reported that anti-clostridial agents were capable of reversing both gastrointestinal and neurological symptoms in one sporadic case of myoclonic-dystonia, which might provide clues to the mechanisms driving dystonia and potentially other neurological conditions with a gut microbial component. However, the relationship between the gut microbiota and isolated dystonia remains poorly explored.

### **Added value of this study**

Our results demonstrated the alterations of gut microbiome in isolated dystonia. The most pronounced high-level correlation was a general overrepresentation of Clostridiales and underrepresentation of Bacteroidetes in dystonia compared with Healthy controls. Furthermore, genes related to the citrate cycle, vitamin B6 and glycan metabolism were less abundant in dystonia, while genes linked to purine and tryptophan biosynthesis were more abundant. Altered levels of several serum metabolites were found to be associated with microbial changes, such as D-tyrosine and glutamate, indicating differences in neurotransmitter metabolism.

### **Implications of all the available evidence**

This is the first study to reveal significant alterations of gut microbiome in isolated dystonia patients. Integrative analysis of microbiome and metabolome suggest that 5-HT system dysfunction may be a possible pathway by which the gut microbiome participates in the development of dystonia. The gut microbiome changes provide new insight on the pathogenesis of dystonia, suggesting new potential therapeutic directions.

## **Introduction**

Dystonia is a complex, highly variable neurological movement disorder characterised by sustained or intermittent muscle contractions<sup>1</sup>. It can be the manifestation of many neurological disorders, either in isolation (isolated dystonia) or in combination with additional symptoms (combined dystonia)<sup>2</sup>. Isolated dystonia includes syndromes in which dystonia is the sole phenotypic manifestation, with the exception of tremor<sup>1</sup>. Until now, the etiology and pathogenesis of isolated dystonia had not been fully elucidated. Most patients with common adult-onset dystonia tend to arise sporadically with no clear etiology, though several associated genes were identified as the cause of childhood- and adolescent-onset dystonia<sup>3</sup>.

The gut microbiome contains a complex community of microbes that live within the gastrointestinal tract. Gut microbes communicate with the central nervous system through nervous-, endocrine- and immune-signalling mechanisms. The brain-gut-microbiome axis allows the brain to control gut function, but also provides an opportunity for the gut microbiome to influence the brain<sup>4</sup>. Emerging evidence has implicated the gut microbiome as an important player in neurodevelopment, as well as in brain diseases such as PD<sup>5</sup>, Alzheimer's disease (AD)<sup>6</sup> and multiple sclerosis<sup>7</sup>. Interestingly, it was reported that anti-clostridial agents were capable of reversing both gastrointestinal and neurological symptoms in one

sporadic case of myoclonic-dystonia<sup>8</sup>, which provides clues to the mechanisms driving dystonia and potentially other neurological conditions with a gut microbial component. However, the relationship between the gut microbiota and isolated dystonia remains poorly explored.

In the present study, we first investigated gut microbial characteristics in drug-naïve patients with isolated dystonia using 16S rDNA amplicon sequencing. We also evaluated whether the parameters correlated with clinically relevant features of dystonia, such as age at disease onset, disease duration, direction of rotation, and dystonia severity. Furthermore, we performed shotgun metagenomic sequencing and non-targeted metabolomics profiling of serum from patients with significant gut dysbiosis in 16S rDNA analysis to investigate the microbial functional alteration and the association of the altered gut microbiome with serum metabolites in the host.

## Methods

### Participants

Fifty-seven patients with isolated dystonia (15 male, 42 female) and 27 age- and sex-matched healthy participants (nine male, 18 female) were enrolled. Among the patients, 44 had focal dystonia, 11 had segmental dystonia, and two had general dystonia. All patients, from 12 provinces across the country, admitted to the Movement Disorders and Botulinum Toxin-A Treatment center underwent a standardised neurological examination by a specialist in movement disorders. All dystonia patients were newly diagnosed and did not receive oral medicine or botulinum toxin injection. None of the participants had a family history of dystonia. All patients were negative for the genetic testing of *DYT1 (TOR1A)*, *DYT4 [TUBB4]*, *DYT6 (THAP1)*, *DYT23 (CIZ1)*, *DYT24 [ANO3]*, *DYT25 [GNAL]* and *COL6A3*. Subjects who have no neurological or psychiatric disease were enrolled as Healthy controls (HCs). Subjects were excluded if they had gastrointestinal diseases, malignant tumours, autoimmune disorders, infectious diseases, a history of gastrointestinal surgery, or were administered antibiotics or laxatives or immunosuppressive agents in the past three months. No subjects were or are on any antianxiety or antidepressant medication. No relevant constipation was present in all subjects; dietary and smoking habits did not differ between groups. No detailed dietary plan was requested prior to the feces collection and samples were collected as first bowel movement of the day.

Demographic information (gender, age, age of disease onset, disease duration, height and weight,) and severity evaluation scores, including Global Dystonia Rating Scale (GDS) and Unified Dystonia Rating Scale (UDRS) were collected from all patients. Evaluation for anxiety and depression including Hamilton Anxiety Scale (HAMA) and Hamilton Depression Scale (HAMD), were assessed for all subjects. Written informed consent was obtained from all individuals. The study was approved by the ethics committees of Beijing Tiantan Hospital.

### Analysis of Fecal Microbiota by 16s rDNA Amplicon Sequencing

Stool samples freshly collected from each participant were immediately transported to the laboratory and frozen at -80°C. Bacterial DNA was extracted and the V4 region of the 16S rRNA gene was amplified by PCR using barcoded universal fusion primers 515 and 806. PCR products were sequenced and for taxonomic assignment. The number of reads in each sample was standardised to 37,666 for further analysis. Dirichlet multinomial mixtures (DMM) modeling examines taxa frequencies and determines how many microbial community states (MCS) exist within a dataset using the DirichletMultinomial<sup>9</sup>. Please refer to the Supplemental Methods section for further details on the laboratory protocols.

### **Functional Analysis of Microbiome by Shotgun Metagenomic Sequencing**

Total DNA from the 26 fecal samples (13 cases and 13 controls) were subject to shotgun metagenomic sequencing. Sheared DNA (350 bp) was used for Illumina library construction and index codes were added to attribute sequences to each sample. Functional annotation was carried out by BLASTP searches against the Kyoto Encyclopedia of Genes and Genomes (KEGG database (e-value  $\leq 1e-5$  and high-scoring segment pair scoring  $>60$ ). For each functional feature (KO in the KEGG database), we estimated its abundance by accumulating the relative abundance of all genes belonging to that feature. Please refer to the Supplemental Methods for further details.

### **Untargeted metabolomics analysis of serum samples**

All serum samples were thawed on ice and a quality control (QC) sample, made by blending equal volumes (20  $\mu$ l) of each serum sample, was used to estimate a mean profile representing all the analytes encountered during analysis. Metabolite extraction was performed by mixing 100  $\mu$ l thawed serum and 400  $\mu$ l ice-cold 80% methanol and 0.1% formic acid, vortexing for 30 s, and keeping at -20 °C for one hour. After centrifuged at 14,000 g and 4 °C for 20 min some of supernatant was diluted to final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 14,000 g, 4°C for 20 min. Finally, the supernatants were subjected to metabolomics profiling by high performance liquid chromatograph (HPLC)-MS in both positive and negative ionization modes. The raw data files were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher). Peak intensities were normalized to the total spectral intensity. All data were mean-centred and unit variance (UV)-scaled before multivariate statistical analysis, and partial least squares discriminant analysis (PLS-DA) was performed. Significantly different metabolites were determined by a combination of VIP value  $>1$  from the PLS-DA model and p-values  $<0.05$  from two-tailed Student's t tests. Please refer to the Supplemental Methods for further details.

### **Spearman's multi-omics correlation analysis**

Spearman's correlation coefficients were computed for relationships between the relative abundance of identified dystonia-associated species and normalised individual metabolomic features. A scaled heatmap was constructed for the correlation matrix, including cladogram classification of variables, using the default clustering method. Visual presentation of multiple omics correlations was performed

using the Corrplot package in R (v3.5.3). Significant microbiota-metabolite correlations were determined based on an  $r$  value  $<0.3$  or  $>0.3$  and a false discovery rate (FDR)-adjusted  $p$ -value  $<0.05$ .

## Results

### Demographics and clinical data

Fifty-seven isolated dystonia patients and 27 HCs were recruited and an integrative analysis of the gut microbiome and serum metabolome was performed. Clinical details of the study cohort are shown in Table 1.

### Data overview

Totally, 4,397,348 high-quality 16S rDNA V4 sequences were obtained, with  $52,349 \pm 8462$  (mean  $\pm$  SD) per sample after de-multiplexing and quality control (Supplementary Table S1). A total of 997 OTUs ( $>97\%$  similarity, excluding singletons and low-abundance taxa with frequency  $<3$  for samples, range = 204–461, median = 306 taxa per sample) were identified, representing 22 taxonomic phyla. The dominant phyla were *Firmicutes* (66.4%), *Bacteroidetes* (17.5%), *Proteobacteria* (9.4%) and *Actinobacteria* (5.3%). At the genus level, *Bacteroides* (13%), *Faecalibacterium* (10%), unidentified *Lachnospiraceae* (7%), *Agathobacter* (6%) and *Blautia* (5%) were the most abundant, of which all but *Bacteroides* belong to *Clostridiales* in *Firmicutes* (Supplementary Figure S1).

### Gut microbiota revealed by 16s rDNA amplicon sequencing

Although gut microbial  $\alpha$ -diversity was not significantly different as calculated by Shannon and Simpson indices (Figure 1A), NMDS analysis demonstrated a significant difference between isolated dystonia and HCs (Figure 1B, PERMANOVA,  $R^2 = 0.157$ ,  $p = 0.002$ ). We also observed higher  $\beta$ -diversity in the microbiota of dystonia patients based on ANOSIM analysis, indicating a more heterogeneous community structure among dystonia patients compared with HCs (Figure 1C). LEfSe analysis showed that *Clostridiales* and *Bacteroidetes* were the key taxa distinguishing isolated dystonia from HCs. Dystonia patients were significantly enriched with *Blautia*, *Bifidobacterium* and unidentified *Lachnospiraceae*, while depleted with *Bacteroidetes* and unidentified *Clostridiales* as compared with HCs (Figure 1D). The relative abundance of the most abundant taxa ( $\geq 1\%$ ) at different taxonomic levels was further compared (Supplementary Table S2 and Figure S2). At the species level, *Eubacterium hallii*, *Blautia obeum* and *Dorea longicatena* were enriched in dystonia subjects, while *Bacteroides vulgatus*, *Bacteroides plebeius* and *Fusobacterium varium* were enriched in HCs (Figure 1E).

### Stratification of bacterial community in dystonia patients

Given more heterogeneity, we applied DMM to our cohort to investigate possible gut microbial subgroups in dystonia, and identified two significantly distinct MCSs ( $n = 8$  and  $n = 49$ ;  $R^2 = 0.138$ ;  $p < 0.001$ ) using a Laplace approximation (Figure 2A). Specific co-occurring bacterial families were characteristically

enriched in these groups; the MCS1 (n = 8) microbiota was characteristically enriched by *Bacteroidaceae*, *Ruminococcaceae* and *Lachnospiraceae*. The second larger group, which we designated MCS2A (n = 20) and MCS2B (n = 29), respectively exhibited a sharp decreased abundance of *Bacteroidaceae*, especially in MCS2B (Figure 2B). NMDS analysis confirmed a strong and significant relationship between MCS class and bacterial  $\beta$ -diversity (PERMANOVA,  $R^2 = 0.29$ ,  $p < 0.001$ ), corroborating the existence of compositionally distinct microbial states (Figure 2C). These distinct microbial states exhibited significant differences in diversity (Shannon, Kruskal-Wallis tests,  $p = 0.002$ ; Figure 2D), with MCS2B exhibiting the lowest index. We also investigated whether specific factors (illness duration, age at onset, BMI and severity evaluation scores) that was differentially associated with microbial community states. No significant correlation was detected.

### Functional analysis of the isolated dystonia by metagenomic sequencing

As shown above, 29 of 57 (51%) the isolated dystonia underwent a gut microbial dysbiosis. To further explore microbial functional feature of dystonia, we performed metagenomic sequencing of a subgroup consisting of 13 patients from MCS2B and 13 gender- and age-matched HCs. An average of  $10.7 \pm 2.1$  Gb clean reads were generated per sample except for one HC sample removed due to low depth of sequencing (Supplementary Table S3).

Consistent with the 16S rDNA analysis, *B. obeum*, *D. longicatena* and *E. hallii* were significantly increased in dystonia, while *B. vulgatus* and *B. plebeius* were decreased (Figure 1F and Supplementary Figure S3). We estimated the abundance of metabolic pathways using metagenomic reads mapped to functional orthologs from the KEGG databases to explore differences in the metabolic potential of gut microbiomes between dystonia and HCs. Dystonia communities were functionally different from healthy communities, and less closely clustered together among individuals, suggesting that inter-individual functional variation was higher in dystonia than in HCs (Figure 3A).

A total of 22 metabolic pathways (level 3) were found to be significantly different ( $p < 0.05$ ,  $q < 0.02$ ) in abundance ( $>0.01\%$ ) between dystonia patients and HCs, including those involved in nucleotide, amino acid, carbohydrate and lipid metabolism (Supplementary Table S4). Interestingly, we identified several different pathways that appeared to be more active in the microbiome of dystonia patients, including purine metabolism (ko00230), peptidoglycan biosynthesis (M00550), glycerolipid/glycerophospholipid metabolism (ko00561, ko00564), phenylalanine, tyrosine and tryptophan biosynthesis (ko00350, ko00400) and sulphur metabolism (ko00920) (Figure 3B). While genes related to TCA cycle (ko00020, ko00630, ko00720), glycan biosynthesis and metabolism pathways (ko00511, ko00531, ko00540, ko00600, ko00603) and vitamin B6 metabolism (ko00750) were less abundant in dystonia (Figure 3C). Furthermore, we traced the contributing genes and determined their likely taxonomic origin to determine which bacteria are involved in these pathways (Figure 3B, C).

### Associations between gut microbial species and serum metabolites

To investigate the extent to which the altered microbiome in the dystonia patients was associated with serum metabolites in the host, we performed non-targeted metabolomics profiling of serum from the 13 dystonia patients and the 12 HCs in metagenomic analysis, and yielded 1543 features (Supplementary Table S5). A supervised PLS-DA using two components ( $R^2X = 0.525$ ,  $R^2Y_{cum} = 0.99$ ,  $Q_{cum}^2 = 0.975$ ,  $p < 0.001$ ) was performed, resulting in some separation tendencies between dystonia cases and HCs (Figure 4A). Based on the PLS-DA models of metabolite profiling data, 242 metabolites were found to be significantly different in abundance between dystonia cases and HCs, with 87 metabolites having a higher concentration and 169 metabolites having a lower concentration in dystonia compared with HCs ( $p < 0.05$ ,  $VIP > 1$  and  $FC > 2$  or  $< 0.5$ , Figure 4B, Supplementary Table S5).

Subsequently, Spearman's correlation coefficients were computed for relationships between the relative abundance of the identified dystonia-associated species and the different 242 normalised individual metabolomic features. Metabolites were grouped into two clusters depending on the correlations, and correlation coefficients with significant  $p$ -values ( $< 0.01$ ) are shown in Figure 4C. *Clostridiales* species enriched in dystonia patients correlated positively with the first metabolite cluster, including L-glutamic acid, phenylalanylphenylalanine and taurine. While *Bacteroides* species with decreased abundance in dystonia patients correlated positively with the second cluster, including D-tyrosine, D-(-)-aspartic acid and N, N-dimethylacetamide (DMAc).

## Discussion

An integrative analysis of the gut microbiome and serum metabolome was employed to explore the relationship between the gut microbiome and isolated dystonia. We found that more than half of the isolated patients (29/57, 51%) got a gut microbial dysbiosis in 16S rRNA analysis, showing a general overrepresentation of *Clostridiales* and underrepresentation of *Bacteroidetes* in dystonia compared with HCs. Further analysis thus for this subgroup using shotgun metagenomic sequencing and non-targeted metabolomics profiling of serum was performed to gain an insight into the links between gut dysbiosis and dystonia, and indicated alterations in several genes and serum metabolites. Genes related to the citrate cycle, vitamin B6 and glycan metabolism were less abundant in dystonia, while genes linked to purine and tryptophan biosynthesis were more abundant. Altered levels of several serum metabolites associated with microbial changes, such as D-tyrosine and glutamate, were found, indicating differences in neurotransmitter metabolism.

Evidence is accumulating for a potential role of the microbiota in the pathogenesis of neurological disorders<sup>5-8</sup>. One myoclonic dystonia case was reported to present a 90% improvement in tremor and abnormal posture after taking anti-clostridial antibiotics, indicating a possible correlation between the pathogenesis of dystonia and the gut microbiome, underlying the potential role of *Clostridial* species<sup>8</sup>. In this study, intestinal dysbiosis was characterised by significantly increased *Clostridiales* and decreased *Bacteroidetes* in dystonia patients, which might explain the neurological improvement in the abovementioned case after taking anti-clostridial agents, suggesting that *Clostridiales* may have a potential pathogenic role in the pathogenesis of dystonia.

*Blautia* and unidentified *Lachnospiraceae*, belonging to the *Lachnospiraceae* family, contributed principally to the increase in *Clostridiales* in dystonia patients. The *Lachnospiraceae* family is known to participate in the breakdown of carbohydrates into short-chain fatty acids (SCFAs)<sup>10</sup>, which are believed to play a key role in microbiota-gut-brain crosstalk<sup>11,12</sup>. SCFAs, with acetate, propionate and butyrate being the most abundant components, may influence the brain by crossing the blood-brain barrier (BBB)<sup>13</sup>, modulating neurotransmission, influencing levels of neurotrophic factors, and promoting serotonin (5-hydroxytryptamine, 5HT) biosynthesis<sup>12</sup>. In a cell model, acetate and butyrate promoted serotonin biosynthesis by inducing the expression of tryptophan 5-hydroxylase 1 (the rate-limiting enzyme for 5HT synthesis) transcription<sup>14</sup>. Another study found that butyrate and propionate play a critical role in promoting host 5HT biosynthesis, and regulating levels of 5HT in both colon and serum<sup>15</sup>. Furthermore, they also induce tyrosine hydroxylase gene transcription<sup>16</sup>, which catalyses the ratelimiting step in the biosynthesis of dopamine, noradrenaline and adrenaline<sup>17</sup>. A decrease in SCFA production can cause intestinal barrier dysfunction, but whether an increased dose of SCFAs could directly lead to adverse effects on locomotor behaviour has not been elucidated and requires animal studies in the future. In our current study, the increased abundance of *Clostridiales* may have led to elevated SCFA levels, and a consequent increase in 5HT.

The abundance of *Bacteroides*, *Parabacteroides* and *Alistipes* was significantly decreased in isolated dystonia compared with HCs. A decrease or low level of *Bacteroides* is believed to be associated with metabolic diseases such as obesity<sup>18</sup> and diabetes<sup>19</sup>, and psychiatric disorders such as depression<sup>20</sup>. Given that no subjects were associated with obesity or diabetes, and there were no differences in body-mass index (BMI) between the two groups, an association between *Bacteroides* and metabolic factors can be ruled out in our study. Growing evidence suggests that non-motor symptoms are essential components of dystonia, with psychiatric co-morbidity being most prevalent<sup>21</sup>, and a link between dystonia and depression has been confirmed<sup>22</sup>. In our cohort, patients were newly diagnosed and there was no statistical difference of HAMD between the two groups, it is unlikely that depression was a confounding factor in our study. *Alistipes* species are indole-positive and may thus influence tryptophan availability<sup>23</sup>. As tryptophan is also the precursor of serotonin, decreased abundance of *Alistipes* might therefore disrupt the balance of the intestinal serotonergic system.

Until now, the pathophysiological basis of dystonia remains poorly understood. Evidence indicates that the pathophysiological basis of dystonia involves dysfunction of the basal ganglia and cortico-striatal-thalamo-cortical circuits<sup>24</sup>, hence neurotransmitter systems are likely involved. In addition to dopamine, a significant neurotransmitter in motor control, serotonin and serotonin-dopamine interactions have received attention<sup>25</sup>. Disturbance of the serotonergic system is known to be involved in extrapyramidal movement and psychiatric disorders. A [<sup>11</sup>c]DASB positron emission tomography study of cervical dystonia patients revealed a significant correlation between serotonin transporter (SERT) binding in the dorsal raphe nucleus and motor and non-motor symptoms<sup>26</sup>. Higher non-displaceable binding potential (BPND) was statistically correlated with motor severity, pain and sleep disturbance, with motor symptom

severity being the most important predictor of SERT binding<sup>26</sup>. Since *Lachnospiraceae* and *Alistipes* are involved in the production and regulation of 5-HT biosynthesis, the increase in the *Lachnospiraceae* family and the decrease in *Alistipes* species observed in our study may lead to imbalance of peripheral levels of 5-HT, which might, in turn, modulate brain function by affecting the immune system<sup>27</sup> or signalling to the brain via 5HT receptors on vagal afferent fibres<sup>28</sup>.

Functional analyses revealed that phenylalanine, tyrosine and tryptophan biosynthesis tend to be more active in dystonia, as reported previously for PD<sup>29</sup>. These aromatic amino acids are important as precursors of 5-HT and catecholamines. Correspondingly, metabolome analysis found that the serum concentration of D-tyrosine, the precursor for serotonin, was significantly elevated in dystonia cases. Composition analysis, functional analysis of the gut microbiome, serum metabolome analysis, and imaging all point to a potential role of serotonergic system dysfunction in the pathogenesis of isolated dystonia.

Additionally, the sulphur metabolism pathway appeared to be more active in the microbiome of dystonia patients. In the brain, sulphur metabolism is intimately involved in metabolic cooperation, and provides cells with four major reagents critical for methylation reactions (S-adenosylmethionine), antioxidant capacity (glutathione), signalling (H<sub>2</sub>S), and cell volume regulation (taurine). An important intermediate in sulphur metabolism is homocysteine (HCY), a neuronal excitotoxic amino acid, and hyperhomocysteinaemia is considered an independent vascular risk factor. Increased HCY has also been observed in movement disorders such as PD<sup>30</sup>, Huntington's disease (HD)<sup>31</sup> and dystonia<sup>32,33</sup>. In patients with isolated dystonia, hyperhomocysteinaemia was observed independently from immune activation<sup>33</sup>. Our study indicates that hyperhomocysteinaemia might, to some extent, be related to an increase in sulphur metabolism caused by disturbance of the gut microbiome.

It should be noted that the diversity of the gut microbiome is influenced by several factors, including health status, age, diet, and antibiotics<sup>34</sup>. One highlight of our study is the enrollment of drug-naïve patients. Evidence is accumulating on effects that drugs can have on the composition and function of the gut microbiome<sup>35</sup>. A case-control study on cause-effect relationships could be performed in the future by analysing drug-naïve dystonia patients.

Despite being, to our knowledge, the first study to explore the alterations of gut microbiome in dystonia, the present study has some limitations that should be acknowledged. First, this study was performed at one single centre (Tiantan Hospital). Selection bias and unknown confounding factors cannot be excluded. However, our hospital has a big center for neurological diseases and appeals to the patients across the country. Patients in our cohort came from 12 provinces and are to some extent, representative. Second, our study is an exploratory study and the number of our patients is relatively small. More studies including larger cohort from different regions and ethics will be needed to confirm the role of gut microbiome in isolated dystonia.

## Conclusions

This is the first study to reveal a significant gut microbial dysbiosis in isolated dystonia patients, which may be associated with altered serum metabolites. Integrative analysis of microbiome and metabolome suggest that 5-HT system dysfunction may be a possible pathway by which the gut microbiome participates in the development of dystonia. The findings provide new insight on the pathogenesis of dystonia, indicating the potential involvement of the enteric neurotransmitter system in dystonic patients. Further validation is needed in animal experiments and in large populations to explore clearer role of gut microbiome in pathogenesis of dystonia.

## Abbreviations

HC: Healthy control; AD: Alzheimer's disease; PD: Parkinson's disease; GDS: Global Dystonia Rating Scale; UDRS: Unified Dystonia Rating Scale; HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale; 16S rRNA: 16S ribosomal RNA; OTUs: Operational taxonomic units; MCS: Microbial community states; DMM: Dirichlet multinomial mixtures; LC-MS: Liquid Chromatography Mass Spectrometry (LC-MS); PLS-DA: partial least squares discriminant analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; SCFA: short-chain fatty acids; 5HT: 5hydroxytryptamine.

## Declarations

### Acknowledgments

We appreciated all the participants in our study.

### Authors' contributions

LY Ma, M Chen and YF Hu participated in the design of the study, collected medical data of the subjects, undertook laboratory work, analyzed the data and drafted the manuscript. Hua Pan collected medical data of the patients. Bo Liu and Jian Yang performed bioinformatic analysis. YF Hu, Tao Feng and Qi Jin carried out the conceptualization of the study.

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### Availability of data and materials

The sequencing dataset has been deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences under BioProject accession code PRJCA003075.

### Ethics approval and consent to participate

All participants provided informed consent and the study was approved by the ethics board of Beijing Tiantan Hospital.

### **Consent for publication**

The paper has not been published previously and is not under consideration for publication elsewhere. All co-authors have read and approved the submission.

### **Competing interests**

All authors declare that they have no competing interests.

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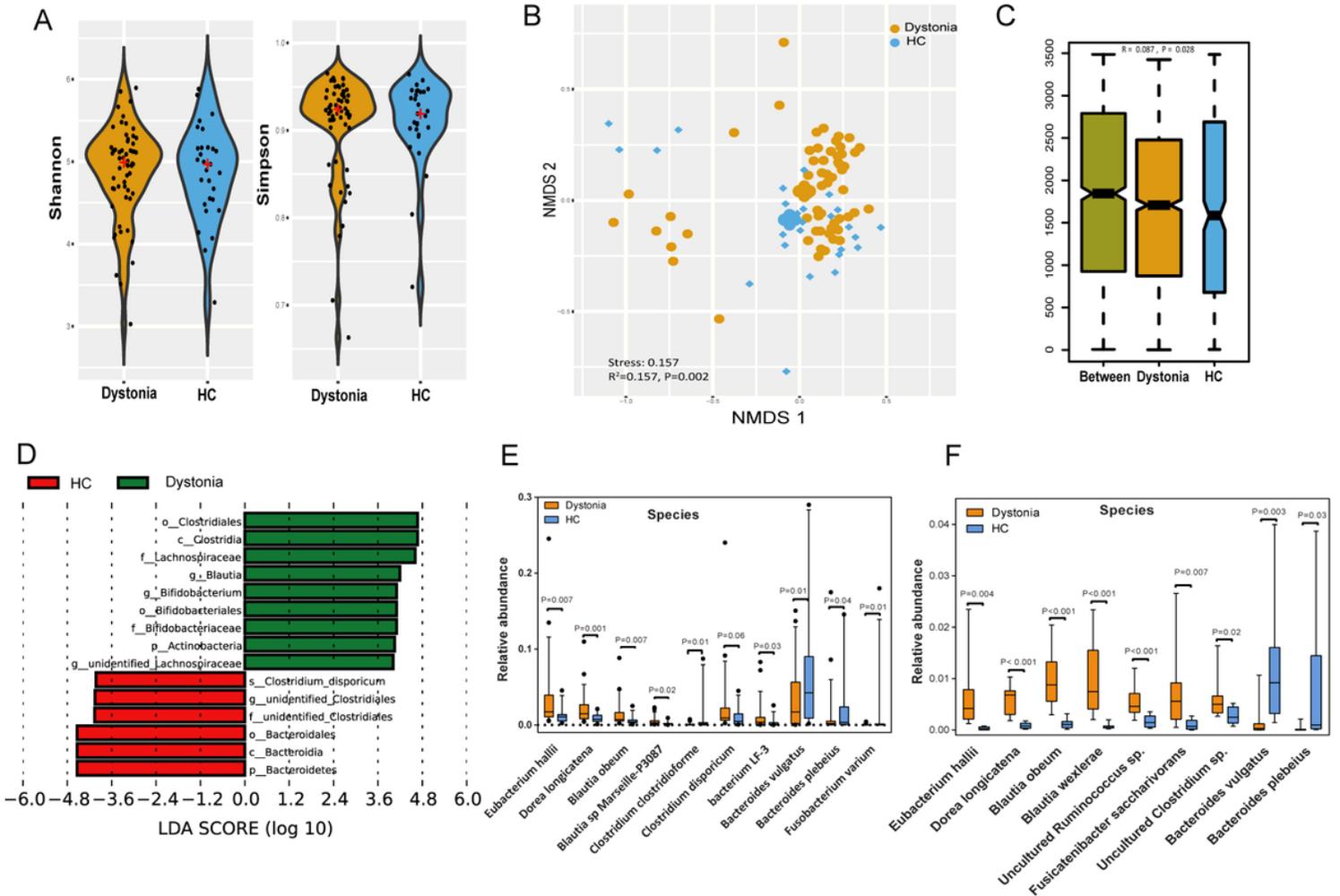
## Tables

**Table 1 Demographics of subjects**

	Dystonia (n = 57)	Control (n = 27)	<i>P</i> value
Male ( <i>n</i> ) (%)	15 (26.32)	9 (33.33)	0.506
Female ( <i>n</i> ) (%)	42 (73.68)	18 (66.67)	
BMI (means ± SD)	22.71 ± 2.04	23.61 ± 1.80	0.341
Age (year; means ± SD; range)	45.25 ± 13.00	41.1 ± 11.15	0.158
Age at onset (year; means ± SD)	45.05 ± 12.86	-	
Duration (month; means ± SD)	5.32 ± 3.77	-	
UDRS (means ± SD)	9.91 ± 14.43	-	
GDS (means ± SD)	6.54 ± 4.06	-	
With family history ( <i>n</i> ; %)	0	-	
HAMA (means ± SD)	8.51± 4.21	6.78± 3.11	0.060
HAMD (means ± SD)	8.14± 4.13	6.52± 2.67	0.066

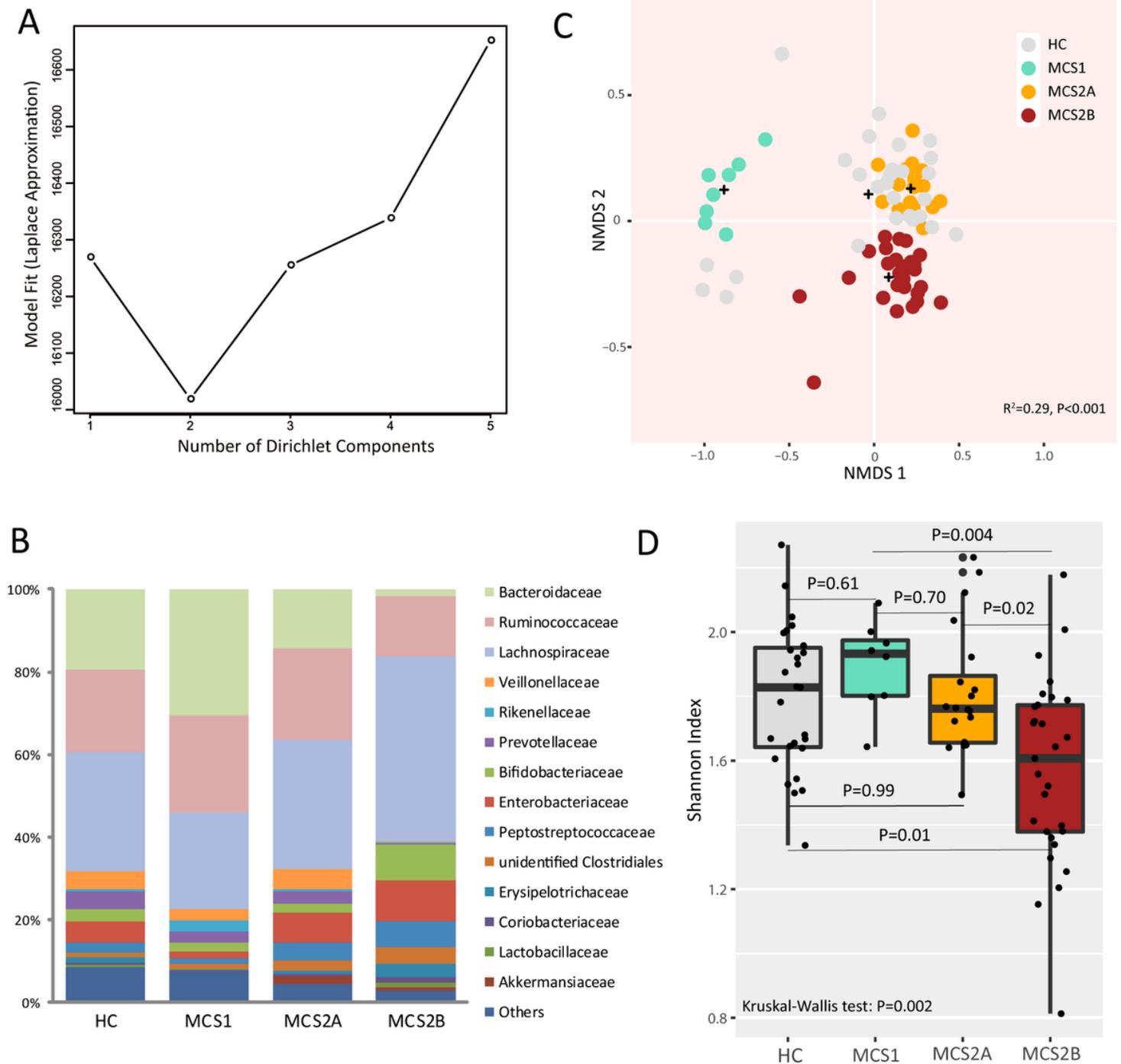
UDRS, Unified Dystonia Rating Scale; GDS, Global Dystonia Rating Scale; HAMA, Hamilton anxiety scale; HAMD, Hamilton depression scale

# Figures



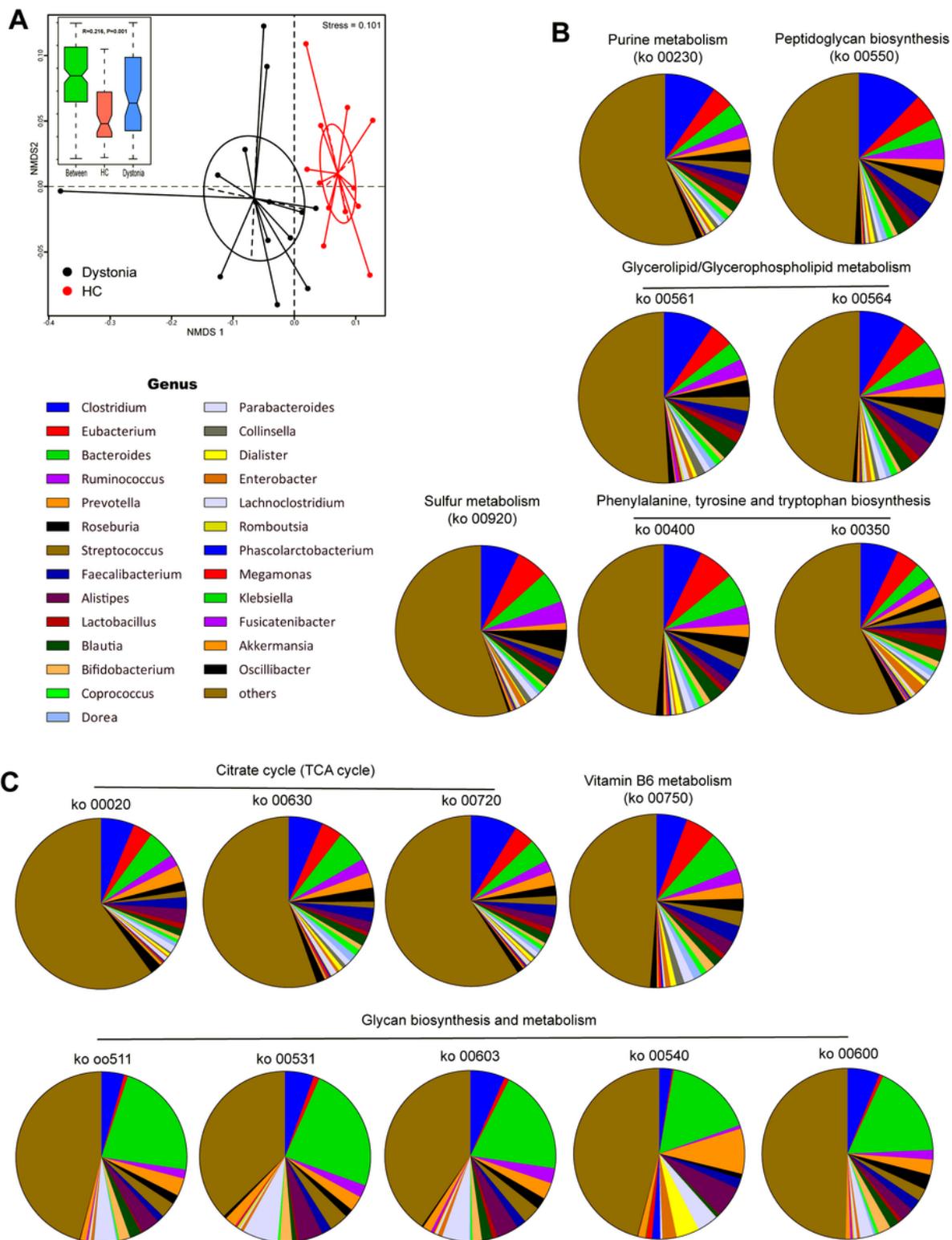
**Figure 1**

Gut microbiome differences between dystonia patients and HCs. (A) Alpha-diversity calculated by Shannon and Simpson indices. Significance was confirmed using Mann-Whitney U tests. (B) Beta-diversity of gut bacterial communities between isolated dystonia patients and HCs using unsupervised ordination (NMDS). Centroids are indicated by crosses. Significant differences were confirmed by PERMANOVA ( $R^2 = 0.157$ ,  $p = 0.002$ ). (C) within-group and between-group dissimilarities analysed by ANOSIM ( $R = 0.087$ ,  $p = 0.028$ ). (D) LeFSE analysis revealing significant taxonomic differences between isolated dystonia patients and HCs. (E) Comparison of the relative abundance of the most abundant taxa ( $\geq 1\%$ ) at the species level in 16S rDNA amplicon sequencing. (F) Comparison of the relative abundance of the most abundant taxa ( $\geq 1\%$ ) at the species level in metagenomic sequencing.



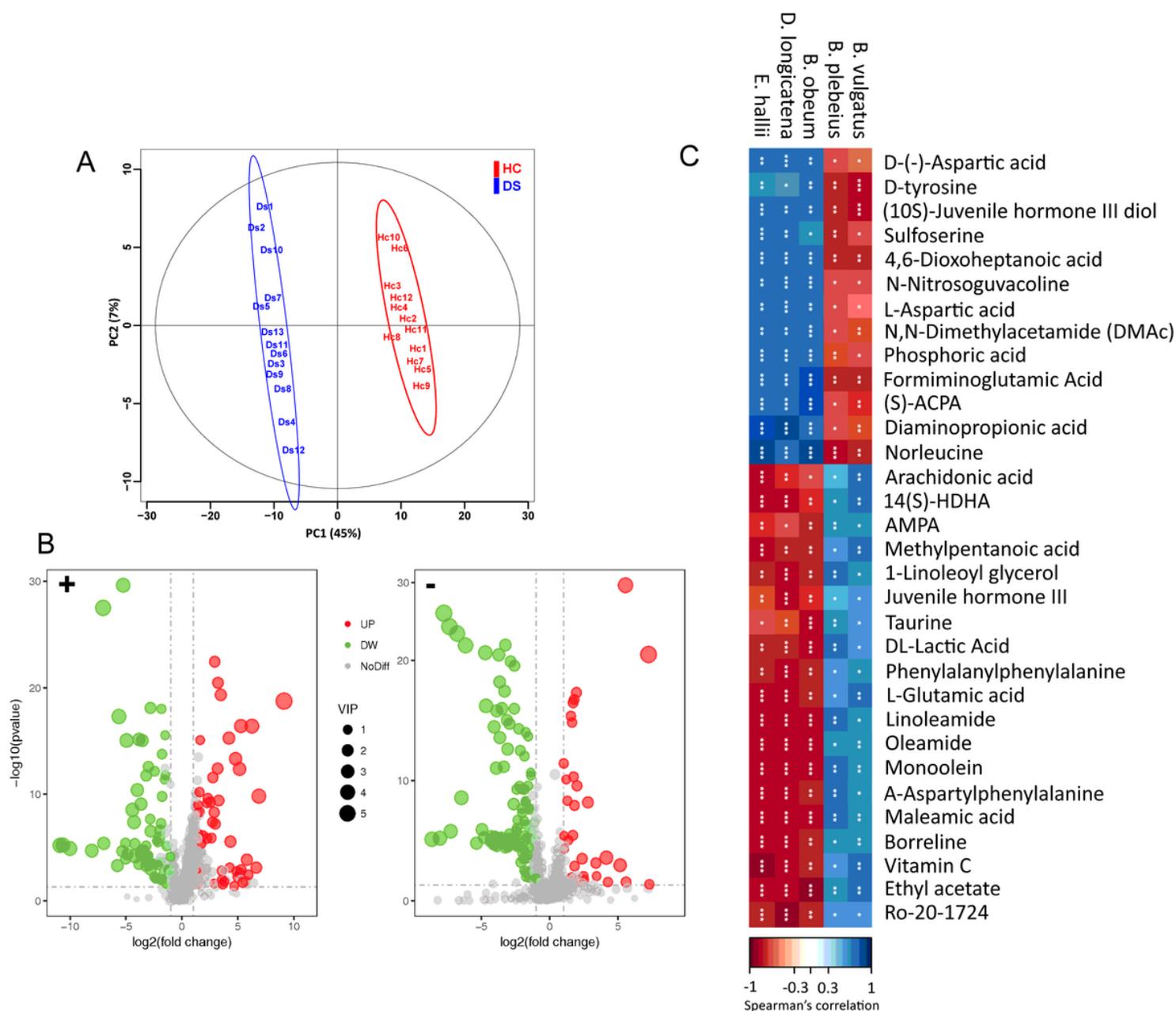
**Figure 2**

Two compositionally distinct gut microbial states exist in isolated dystonia patients. (A) Based on Laplace approximation, Dirichlet Multinomial Mixture (DMM) analysis identified two compositionally distinct bacterial communities ( $n = 8$  and  $n = 49$ ) in the gut of patients with isolated dystonia. (B) Mean community composition of each microbial state at the family level. (C) NMDS analysis illustrates that DMM-defined gut bacterial communities are compositionally distinct (PERMANOVA,  $R^2 = 0.29$ ,  $p = 0.001$ ). (D) Shannon diversity differs significantly across microbial states (ANOVA,  $p < 0.001$ ).



**Figure 3**

Functional characterisation of genes related to dystonia. (A) Functional characterisation of the dystonia microbiome. (B, C) NMDS plot of Bray-Curtis resemblance generated from square root-transformed KEGG pathway (level 3) relative abundances. Functional differences in dystonia patients are based on selected metabolic pathways via genus-level composition of 16 modules increased (B) or decreased (C) in isolated dystonia patients compared with HCs.



**Figure 4**

Associations of gut microbial species with circulating metabolites. (A) Supervised partial least squares discriminant analysis (PLS-DA) showing differences between dystonia patients and HCs. (B) Volcano plot of metabolic features detected in serum samples after background subtraction and removal of the features found in <30% of the data. Positive  $\log_2$  fold change (FC) indicates increased abundance in isolated dystonia patients; '+' indicates positive ionisation mode and '-' indicates negative ionisation mode. All p-values were adjusted using the Bonferroni method. (C) Heatmap of Spearman's rank correlations between the five bacterial species displaying altered abundance in dystonia, and 242 different metabolites (only metabolites correlated with at least one species with adjusted  $p < 0.01$  are shown; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

## Supplementary Files

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