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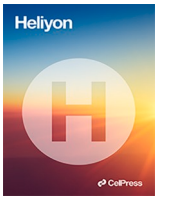

Research article

Therapeutic effects and mechanisms of fecal microbiota transplantation on EAE partly through HPA axis-mediated neuroendocrine regulation

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A R T I C L E I N F O

Keywords:
Fecal microbiota transplantation
Multiple sclerosis
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Hypothalamic-pituitary-adrenal

A B S T R A C T

Background: The pathogenesis of multiple sclerosis (MS) may be closely related to immune regulation and inflammatory cytokines induced by specific flora. Repairing the intestinal flora may alter the immune response in MS patients, thus opening up novel approaches for the treatment of MS.
Objective: We aimed to test the therapeutic effect of fecal microbiota transplantation (FMT) on experimental autoimmune encephalomyelitis (EAE) and the characteristics of intestinal microbiota composition changes, explore the potential mechanisms of FMT treatment.
Methods: EAE animals were treated with FMT, with the therapeutic effects were evaluated by observing neurological scores and measuring serum levels of cortisol, IL-17, and TLR-2. Fecal microbiome 16S rRNA sequencing was used to profile changes in microbiota composition, and adrenalectomy pretreatment was used to test whether FMT effects were dependent on HPA axis function.
Results: FMT improved neurological function and reduced serum IL-17 to levels that were close to the control group. FMT reestablished intestinal homeostasis by altering the structure of the intestinal flora, increasing the abundance of beneficial flora, and regulating intestinal metabolites. We found that the therapeutic effects of FMT depended partly on the efferent function of the HPA axis; surgical disruption of the HPA axis altered the abundance and diversity of the intestinal flora.
Conclusion: FMT showed a neuroprotective effect on EAE by increasing the abundance of the beneficial flora, rebuilding intestinal homeostasis, reducing IL-17 and cortisol serum levels, and promoting serum TLR-2; the therapeutic effect of FMT on EAE is partly dependent on the HPA axis.

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Multiple sclerosis (MS) is a neurological autoimmune disease characterized by inflammation-driven demyelination of the white matter of the central nervous system. The pathogenesis of MS is complex and has high rates of relapse and disability. Specific treatments for MS are currently lacking, and the available therapeutics are burdened by significant side effects. The clinical challenge of managing frequent relapses remains unresolved. Consequently, there is an urgent need to develop new, safe, and effective treatments for MS.

In recent years, numerous authoritative studies have reported a strong association between intestinal microecology and CNS diseases[1,2]. Intestinal flora have been found to form a neuroendocrine network through the CNS, autonomic nervous system, hypothalamic-pituitary-adrenal (HPA)[3] axis, and enteric nervous system. This is also referred to as the microbiota-gut-brain (MGB) axis, which can have a bidirectional influence over the CNS through immune, neuroendocrine, and vagal pathways. The gut flora in MS patients markedly differs from healthy individuals[4] and can inhibit the production of anti-inflammatory molecules such as IL-10[5], which is considered to be the initial inflammatory mediator of demyelination in MS[6]. Thus, the CNS autoimmune inflammatory response in MS may be closely linked to abnormal alterations in intestinal microecology; repairing the intestinal flora may positively alter the immune response in MS patients.

Fecal microbiota transplantation (FMT) involves the transplantation of functional flora from healthy human feces into the patient's gastrointestinal tract, with the aim of re-establishing a functioning intestinal flora for the treatment of intestinal and extraintestinal diseases. FMT was initially shown to be safe and effective in the treatment of *Clostridium difficile* infections and inflammatory bowel disease. In recent years, a growing number of animal and clinical studies have shown that FMT is effective in the treatment of neurological disorders such as autism, Alzheimer's disease, Parkinson's disease, stroke, epilepsy, depression, optic neuromyelitis optica, and MS. One clinical study found[7] that MS patients treated with FMT had improved symptoms; additional studies found significantly higher levels of intestinal immune markers in the blood of treated patients, compared to healthy individuals. Tankou et al. [8] found that oral probiotics also improved clinical symptoms in MS patients. Borody et al.[9] used FMT to treat three MS patients with chronic constipation for up to 15 years, finding a gradual improvement in neurological symptoms and recovery of walking ability in all cases; two patients regained urinary function and the progress of one patient's condition was halted. Al et al.[10] conducted a randomized controlled clinical trial and found FMT to be safe and tolerable in the treatment of MS. Based on these data, FMT has good potential for the treatment of MS.

However, the exact mechanisms of action underlying the clinical effects of FMT in the treatment of MS remain unclear. Elucidating these mechanisms of action will provide an experimental basis for the development of novel, safe, and effective treatments for MS. Studies have confirmed that altered activity of the HPA axis was implicated in the pathogenesis and progression of MS [11]. Changes in the intestinal flora can stimulate both the enteric nervous system and activation of the HPA axis [12,13]. Consequently, we speculate that FMT can ameliorate MS symptoms by rebuilding intestinal homeostasis, regulating immunity and neuroendocrine through HPA axis. In this study, we aimed to verify the therapeutic effect of FMT on the experimental autoimmune encephalomyelitis (EAE) animal immune model of MS and test the concomitant alteration of intestinal flora composition. In addition, we investigated whether FMT mediates the therapeutic effect of neuroendocrine regulation on EAE through the HPA axis and its possible mechanism of action. Our main goal is to generate novel therapeutic ideas to improve the therapeutic approach to MS.

1. Materials and methods

1.1. Experimental animals

Healthy inbred female C57BL/6 SPF grade mice, 5–6 weeks old, weighing about 16–18 g, were purchased from Guangdong Medical Laboratory Animal Centre, China. Female Wistar rats of SPF grade, weighing approximately 180–220 g, were purchased from the Experimental Animal Centre of Southern Medical University, China. All animals were acclimatized for 1 week before experimentation. The animals were housed under pathogen-free (SPF grade) conditions at the Experimental Animal Centre of the First Affiliated Hospital of Guangdong Pharmaceutical University, fed with SPF grade chow (provided by Guangdong Medical Laboratory Animal Centre, China) and *water ad libitum*, with regular replacement of drinking water, chow, and bedding. Natural ventilation and a hygienic and clean feeding environment were carefully maintained. All the animal experiments were performed in accordance with institutional animal ethics guidelines, and following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Guangdong Pharmaceutical University.

1.2. Experimental groups and treatment protocols

Part 1: Test the therapeutic effects of FMT on EAE and the efficacy of FMT from the perspectives of nerve damage and cytokines.

EAE was induced in twenty C57BL/6 mice using myelin oligodendrocyte glycopeptide 35–55 (MOG_{35-55}) (Tocris Bioscience); these were randomly divided into the FMT treatment (FMT) group and the EAE model control (MC) group ($n = 10$ per group). Ten healthy mice of the same age were kept under the same environmental conditions as part of the normal control (NC) group. The FMT group was given a 0.2 ml dose of fecal filtrate prepared from healthy mice by gavage for 5 consecutive days after the peak disease onset and stabilization (no change in neurological function score for 2 consecutive days). The MC and NC groups were provided equal amounts of saline as a placebo gavage treatment in the same manner. The general animal condition including hair loss, appetite, body weight, urination and defecation, and movement were observed daily; changes in body weight were measured and recorded, and neurological function scores were assessed and recorded. One week after the FMT treatment was completed, fecal samples were collected from each

mouse for microbiome 16S rDNA sequencing. Subsequently, venous blood was collected via a retroorbital approach and serum IL-17 was measured by ELISA (R&D Systems).

Part 2: Adrenalectomy pretreatment to test if the therapeutic effect of FMT is dependent on the HPA axis.

Wistar rats were randomly divided into a surgery group, sham-operated group, and normal control (NC) group. The surgery group received bilateral adrenalectomy pretreatment while the sham-operated group only received a comparable incision and suture treatment. The normal control group was not treated. The EAE model was established 7 d after recovery from surgery as follows. Fresh guinea pig spinal cord was prepared as a homogenate and added to complete Freund's adjuvant (CFA) to make an antigenic emulsion, which was inoculated under the foot pads of the limbs of female Wistar rats to establish the EAE model.

The surgery group was then subdivided into the FMT-surgery (FMT-S) group and surgery control (SC) group. Similarly, the sham-operated group was divided into the FMT treatment (FMT) group and model control (MC) group, n = 6 per group. Feces from each group of rats were collected before treatment. FMT was administered for 5 consecutive days after the peak of EAE disease onset and stabilization. The same daily dose of saline gavage was administered to the SC, MC, and NC groups.

The general condition of the animals was observed daily including hair loss, appetite, body weight, urination and defecation, and movement. Body weight was measured and recorded, and the neurological function scores were also assessed. At the peak onset time of EAE after adrenalectomy and immunization, two rats were chosen randomly to abstract spinal cord, which were identified by HE staining and Luxol fast blue myelin staining, in order to verify whether the pathological changes after inoculation in the adrenalectomized rats met to the histological criteria of EAE. Fecal specimens were collected from each group of rats at the end of the FMT treatment. The baseline and post-treatment fecal specimens were analyzed using microbiome 16SrDNA sequencing. In addition, blood was collected from the abdominal aorta and serum was extracted. The levels of serum cortisol, IL-17, and toll-like receptor 2 (TLR-2) were measured by ELISA (R&D Systems).

1.3. Experimental methods

1.3.1. EAE induction

1.3.1.1. Mouse EAE model. EAE mice were induced according to our previous experiment and reference[14,15]. MOG_{35-55} was diluted to 300 μg/ml with 0.01 mol/L phosphate buffer saline (PBS), followed by the addition of an equal amount of CFA (B. tuberculosis at a final concentration of 4 mg/ml). This was stirred and mixed and pumped with a sterilized stirrer to a water-in-oil state to make an antigenic emulsifier for EAE induction, which was placed on dry ice and set aside. Mice were then anesthetized with 0.1 % sodium pentobarbital 0.1 ml by intraperitoneal injection, and the prepared antigenic emulsifier was injected via subcutaneous injection on both sides of the mice's dorsal spine region (four points per animal, 50 μL per site). 500 ng of pertussis toxin (PTX) (Enzo Life Sciences) was injected intraperitoneally at 0 h and 48 h after immunization, and a second booster immunization with equal doses of MOG/CFA was performed 7 d after the first successful immunization.

1.3.1.2. Rat EAE model. EAE rats were induced according to our previous experiment and reference[16,17]. To obtain spinal cords, guinea pigs were sacrificed by an intraperitoneal injection overdose of sodium pentobarbital. The whole spinal cord was quickly pushed out of the spinal canal using a sterile syringe, on ice. The piamater was carefully peeled off using ophthalmic forceps, the cord was weighed, and an equal volume of pre-cooled saline was added. This mixture was thoroughly homogenized using an electric tissue homogenizer in an ice bath to make a 50 % (w/v) guinea pig spinal cord saline homogenate (GPSCH). Immediately, the homogenate was frozen at −20 °C for 10 min, then removed, allowed to thaw naturally at room temperature, then re-frozen at −20 °C. These freeze-thaw steps were repeated 5 times to achieve sufficient cell fragmentation. Finally, the prepared guinea pig spinal cord saline homogenate was stored at 4 °C until use. Equal amounts of GPSCH and CFA were then added to two sterile glass syringes, connected with silicone tubing, and mixed using the double push method, on ice, while applying a fixator to clamp the silicone tubing to the head end of the syringe to avoid extravasation or loss of the emulsion during the operation. Once emulsified, the product was stored at 4 °C and used within 12 h.

Before injection, animals were anesthetized intraperitoneally with 1 % sodium pentobarbital, then approximately 0.1 ml of the antigenic emulsion was injected into each of the four pads of the feet, for a total of 0.4 ml.

1.3.1.3. EAE onset presentation and neurological score. Animals from both experiments were observed daily from day 0 after immunization for their general condition, including hair, feeding, continence and mobility, and body weight; these were measured and recorded. They were also scored for signs of neurological damage according to the following criteria[18]: 0, no detectable signs of EAE; 0.5, limp distal tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of both forelimbs and hind limbs, and 5, death. Animals with a score $\geq$1 were included in the EAE model while dead animals were excluded from all analyses.

1.3.2. Adrenal pre-excision in rats

After acclimatizing, the rats were fasted and their dorsal hair was shaved 1 day before the procedure. They were then anesthetized with 1.0 % sodium pentobarbital 1 ml by intraperitoneal injection. The rat was disinfected with iodophor 3 times at the midpoint of the spine, the skin on both sides was retracted with forceps, and an 0.5–1 cm incision was made along the top and bottom. The skin was

again picked up on each side with forceps and carefully separated from the subcutaneous tissue and mucosa with tissue scissors. Next, using tissue scissors, we cut along both sides of the spine under the costal margins to reveal the kidney with the adrenal gland; the latter was gently picked up and bluntly separated with ophthalmic forceps, without damaging the kidney. Finally, the skin was sutured and observed for bleeding and, if necessary, Yunnan Baiyao was used to stop the wound bleeding. Both adrenal glands were removed via this protocol. After surgery, the rats were kept warm in a thermostatic water bath, and 5 % physiological saline (containing 1 % glucose) was used as a substitute for drinking water. EAE was induced after 7 d of recovery.

1.3.3. Fecal filtrate preparation and the FMT method

We prepared the faecal filtrate by following the fecal extraction method as published before[19,20]. In short, fresh feces from 15 mice were collected on the same day and weighed (SPF-grade normal mice or rats provided healthy faecal bacteria). The suspension was homogenized by adding saline at 1:5 (W/V) and large particles were filtered out by using a gauze net; the filtrate was then collected in a 10 mL centrifuge tube and centrifuged at 2000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended with an equal amount of saline, mixed, then centrifuged and resuspended three more times. The final precipitate was dissolved in 0.9 % saline to form the fecal filtrate which was stored at −80 °C.

The dosage of FMT administration was determined to be 0.2 mL/d of fecal filtrate for mouse and 2.0 mL/d for rats, considering the clinical dosage of human fecal filtrate (1.67 g/kg/d) and the average body weight of mice (20 g) or rats (200 g)[19]; [20]. The fecal filtrate was administered via oral gavage for 5 consecutive days, starting from the day following the peak and stable onset of EAE (i.e. no change in neurological function scores for 2 consecutive days). The EAE model control group and the normal control group were provided equal amounts of saline oral gavage as a placebo.

1.3.4. Fecal sampling

Fecal samples were collected 1 week after the end of the FMT treatment. Autoclaved filter paper was placed in a clean box and the rodents were placed on the filter paper. Immediately after the rodents had defecated, the freshly formed fecal pellets were collected from the paper and placed in sterilized 2 ml tubes with clean forceps. We collected approximately 5 pellets per animal, which were immediately stored on dry ice. The box was disinfected with alcohol and the filter paper was changed after each fecal sample was collected. All samples were stored at −80 °C until analysis.

1.3.5. Fecal DNA extraction and microbiome 16S rRNA sequencing

Total microbiome DNA was extracted from mouse feces using an PowerFecal DNA Kit (Qiagen) according to the manufacturer's protocol. Amplification of the V3+V4 region of 16S rDNA (468bp) was done by using specific primers (338F: 5′-ACTCCTACGG-GAGGCAGCAG-3'; 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) and recovery and purification of PCR products and fluorescence quantification. Sequencing libraries were constructed using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) that accompanies the sequencer. PE150bp sequencing was performed according to the Illumina Miniseq sequencer operating instructions.

Subsequent analysis of alpha diversity indices including ace, Observed_otus, Pielou_e, Shannon, Faith_pd, and Simpson diversity indices, among others, was conducted using QIIME (Quantitative Insights into Microbial Ecology) on the normalized data. Beta diversity analysis based on weighted unifrac distances was also performed. The results were visualized using R software (Version 2.15.3). To assess the clustering of samples in the study, a significant test was conducted using one-way analysis of similarities (Anosim) based on the unifrac phylogenetic distance. Based on unweighted unifrac distance, Mothur software was used to calculate Ranks for the distance between pairs of samples. The group difference is obtained by comparing the mean value in between-group and within-group. Mothur is available from the project website (http://www.mothur.org) as a Windows-compatible executable or as source code for compilation in Unix/Linux or Mac OS X environments. The significance of Anosim is to test whether the differences in between-groups are greater than that in within-groups, so as to determine whether such groups make sense.

Principal Coordinate Analysis (PCoA) was utilized to analyze visualize the complex, multidimensional data. A distance matrix based on weighted unifrac distances was generated for the samples. This distance matric was then transformed into a new set of orthogonal axes through PCoA, where the first principal coordinate represents the maximum variation factor, the second principal coordinate represents the maximum the second principal coordinate, and so on. The PCoA analysis was visualized using various R packages, including WGCNA, stat, and ggplot2, in R software.

To generate operational taxonomic units (OTU), QIIME2 built-in vesearch software (v2.3.4, https://github.com/torognes/vsearch) was used to reduce noise and redundancy for all effective tags sequences of all samples, the signature sequence with frequency 1 was removed, and the OTU and its representative sequence were obtained by denovo clustering method. Compare the OTU representative sequence to the Silva 138.1 database (https://www.arb-silva.de/), and obtain the annotation results using the classify-sklearn algorithm. Python scripts were used to analyze the number of species and annotation information for each sample, and statistical analysis and plotting were subsequently performed. R software was used to draw Heatmap of relative abundance for each taxonomic class and Venn map of common/exclusive species.

To identify differentially abundant bacterial taxa among groups, the linear discriminant analysis (LDA) effect size (LEfSe) analysis was employed, which is combined by linear discriminant analysis with Kruskal-Wallis non-parametric test and Wilcoxon rank test. Only taxa with a log LDA score >3.5 were considered significant.

For analyzing both 16S rRNA gene relative abundances and the predicted metabolic data, scripts provided by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) were utilized, which is a tool for predicting bacterial metabolic function (https://github.com/picrust/picrust2/wiki). Functional predictive analysis is to compare the 16S amplicon

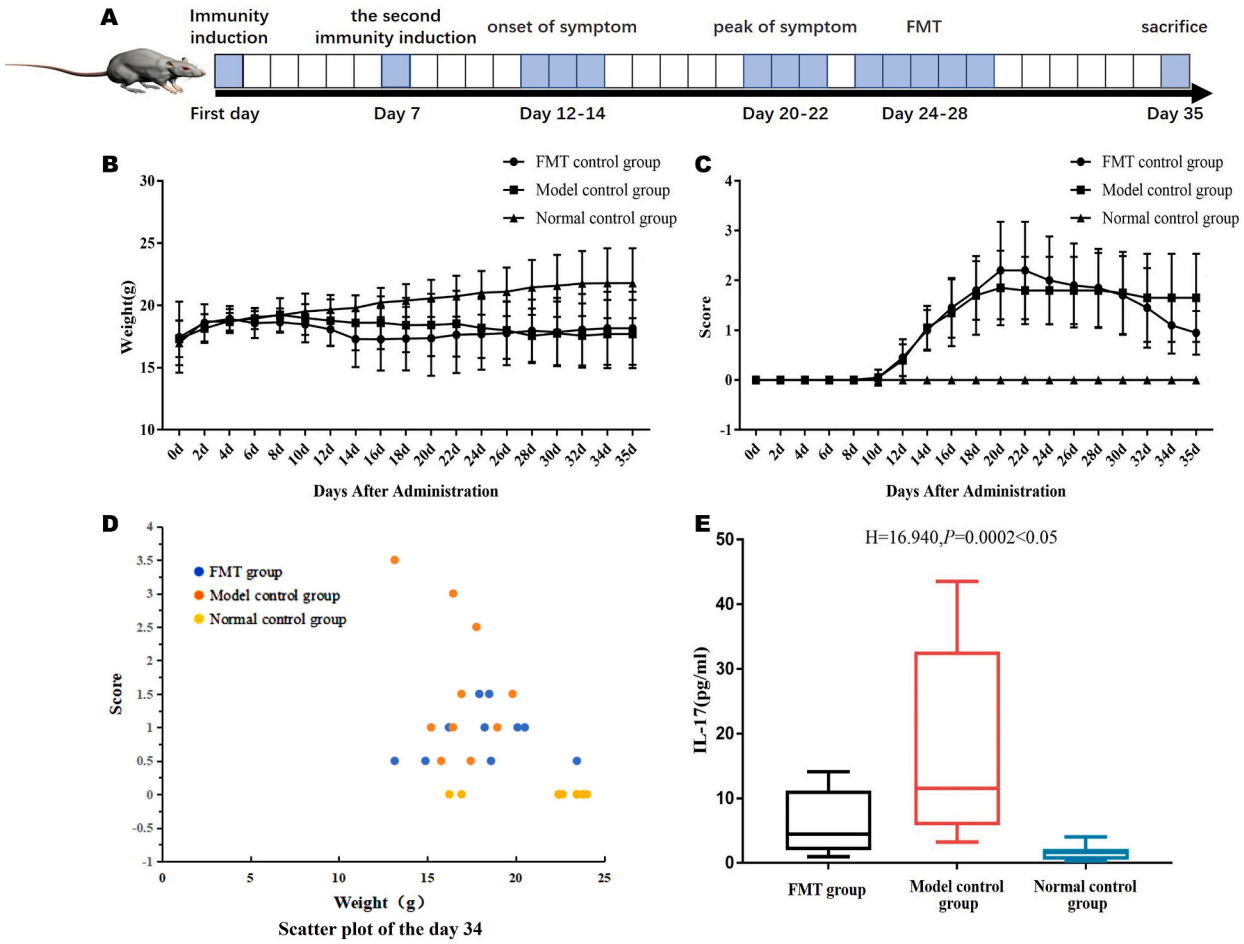

Fig. 1. FMT has protective effects in EAE.
A: EAE experimental flowchart. B: Body weight of mice in different group after administration. The FMT and MC groups displayed progressive weight loss after approximately day 8, after treatment, the FMT group showed a gradually increasing body weight while the MC group continued to decrease. C: Neurological scores in different group after administration. The general trend was that the FMT group was lower than the MC group. D: Scatter plot showed the weight and EAE score of each animal in day 34. E: Levels of serum cytokine IL-17 in different group. MC group showed higher levels of serum IL-17, while FMT group decreased significantly and approached that of the NC group. $p < 0.05$ indicates a significant difference between groups (Kruskal-Wallis non-parametric test, $H = 16.940$, $p = 0.0002$).

sequence with the 16S sequence database of known functional bacteria, to obtain the distribution of known species in the sample, combined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) function information of known species, the functional information and relative abundance of microflora in the sample can be obtained.

1.3.6. Serum cortisol and cytokine assays

The serum samples were quantitatively analyzed by sandwich enzyme-linked immunosorbent assay for cortisol, cytokine IL-17 and TLR-2. The protocol of ELISA kits (R&D Systems, Inc) was followed for the quantikine analysis of cortisol (catalog no. KGE008B), IL-17 (mouse: catalog no. M1700; rat: catalog no. MAB84101) and TLR-2 (catalog no. NBP2-76475). Absorbance was read at 450 nm in an automated ELISA plate reader. A standard curve of optical densities versus concentrations of cortisol, IL-17 and TLR-2 was generated to determine their concentrations in serum samples.

1.4. Statistical analysis

We used SPSS 23.0 (IBM, Armonk, New York, USA) to analyze the data. Plots were generated with Graphpad Prism 7.0. For normally distributed data, groups are summarized by means±standard deviations and differences were tested using one-way ANOVAs (two-way comparisons using the LSD method). Differences within groups were tested by using paired samples t-tests. For non-normally distributed data, the Kruskal-Wallis non-parametric test was used. Correlations were tested using the Spearman's rank correlation analysis, with the correlation coefficient expressed as r. All analyses were two-sided and $p < 0.05$ was considered to indicate statistical

Table 1
Contrast of neurological scores in EAE.

Group	n	Neurological scores		
		Before treatment	After treatment	Score changes
FMT	10	2.40 ± 0.81	0.90 ± 0.39[a]	1.50 ± 0.23[b]
MC	10	2.00 ± 0.82	1.65 ± 0.94	0.35 ± 0.11[b]

Compared to pre-treatment,
[a] $p < 0.001$ (paired samples *t*-test, degrees of freedom was 9, $t = 10.062$); FMT versus to MC group.
[b] $p < 0.001$ (independent-samples *t*-test, degrees of freedom was 18, $t = 14.264$).

significance.

2. Results

2.1. Part 1

2.1.1. FMT shows a protective effect in the EAE mouse model

EAE mice were first immunized at day 0 for induction, second immunized 7 day later, and gavage intervention treatment was started at day 24–28 (Fig. 1A). Before EAE induction, there was no significant difference in body weight among the three groups. The FMT and MC groups displayed progressive weight loss after approximately day 8. After gavage treatment, the FMT group showed a gradually increasing body weight while the MC group continued to decrease (Fig. 1B–D). In addition, compared with the MC group, the FMT group showed significantly better clinical symptoms and lower neurological scores ($p < 0.001$) (Fig. 1C–D, Table 1).

2.1.2. FMT reduces the IL-17 levels

Compared to the NC group, the MC group showed higher levels of serum IL-17 ($p < 0.001$). Whereas, compared to the MC group, the levels of IL-17 in the FMT group decreased significantly ($p < 0.001$) and approached that of the NC group (Fig. 1E).

2.1.3. FMT regulates intestinal metabolites and restores homeostasis by modifying the gut flora, enhancing beneficial bacteria, and regulating metabolites

① Analysis of the species diversity of the intestinal flora

Alpha diversity analysis revealed no significant differences in intestinal flora species diversity among the FMT, MC, and NC groups. (Fig. 2A–D). Beta diversity analysis showed significant cluster separation at PC1 67.6 % on principal components analysis (PCA) ($p = 0.022$) (Fig. 2E–F). Additionally, there was a significant difference in PCo2 16.1 % on PCoA ($p = 0.0025$) (Fig. 2G–H), indicating differences in species diversity in the intestinal flora among the three groups of mice. OTU cluster analysis using the beta-diversity distance matrix showed that the FMT group exhibited a closer similarity to the NC mice in terms of OTU levels (Fig. 2I). The Wayne diagram further quantifies the number of common and unique intestinal flora among the three groups of mice (Fig. 2J). Anosim and MRPP analyses were performed to assess the differences in the structural composition of the bacterial community among the groups. The results indicated significant differences in community composition between the FMT and NC groups (Anosim analysis: $p = 0.006$; MRPP analysis: $p = 0.007$), and between the MC and NC groups (Anosim analysis: $p = 0.015$) (Table 2).

② Analysis of intestinal flora species abundance

To understand the relative abundance of species at each taxonomic level in each sample, the relative distribution of species at each level was demonstrated using R software. Analysis of the intestinal bacterial taxa revealed that in comparison to the MC groups, the FMT group exhibited a lower trend in the abundance of Bacteroidetes, while Verrucomicrobia and Firmicutes showed higher abundance trend, with an increased trend in Verrucomicrobia. Furthermore, when compared to the NC group, the MC group displayed a higher trend in the abundance of Proteobacteria, while Firmicutes exhibited a lower abundance trend (Fig. 3A–B).

③ Analysis of inter-group species differences

To identify the bacterial taxa that were enriched by the differences between the groups, the intestinal flora profiles of the three groups of mice were analyzed using LefSe. We found that, compared to the MC group, in the FMT group, the abundance of Akkermansia muciniphila (A. muciniphila) was significantly higher ($p = 0.042$) while Bacteroidaceae ($p = 0.012$) and Bacteroides acidifaciens ($p = 0.025$) were lower. Muribaculaceae were more abundant in the NC group compared to the MC and FMT groups ($p = 0.016$) (Fig. 4A–C).

④ Correlation analysis of species relative abundance and the severity of EAE in the FMT group

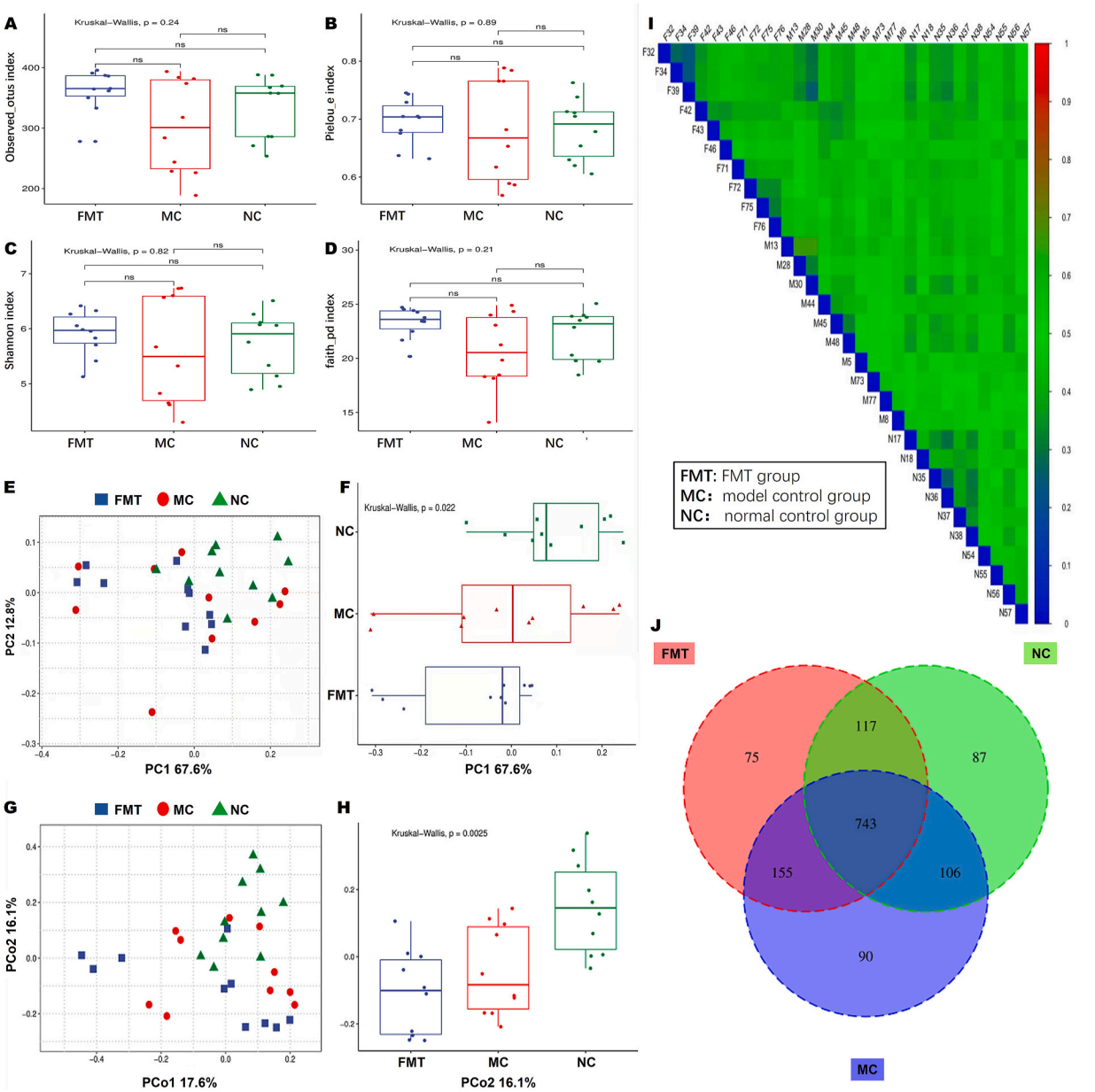

Fig. 2. Analysis of the species diversity of the intestinal flora.
A-D: Box diagram of intestinal flora groups with four indices of α-diversity including Observed_Otus, Pielou_E, Shannon, and Faith_PD. Each color represents a group, and each dot represents a sample. There were no significant differences in intestinal flora species diversity between the FMT, MC, and NC groups. $p < 0.05$ indicates a significant difference between groups, while ns indicated no significant difference between groups. (Kruskal-Wallis non-parametric test, A, $p = 0.24$; B, $p = 0.89$; C, $p = 0.82$; D, $p = 0.21$). E-J: The β-diversity analysis indicates species diversity in the intestinal flora between the three groups of mice. E-F: Significant clustering separation of the three groups of samples at PC1 67.6 % on PCA (Kruskal-Wallis non-parametric test, $p = 0.022$). G-H: Significant clustering separation of the three groups of mice at PCo2 16.1 % on PCoA (Kruskal-Wallis non-parametric test, $p = 0.0025$). I: FMT group was closer to NC mice in terms of OTU levels. Unweighted unifrac distance heat map: each square corresponds to the distance between two samples and the color corresponds to the distance; the smaller the value, the smaller the difference in species diversity between the two samples. J: Wayne diagram quantifies the number of common and unique intestinal flora among the three groups of mice, each colored circle represents a group, the intersecting parts represent the number of species shared by multiple groups, and the non-intersecting parts represent the number of species unique to a single group.

Table 2
Analysis of differences between Anosim and MRPP groups.

Group	Anosim analyses		MRPP analyses			
	R-value	*P*-value	A	Observed-delta	Expected- delta	*P*-value
FMT-MC	−0.025	0.577	−0.007	0.424	0.421	0.561
FMT-NC	0.193	0.006[a]	0.064	0.344	0.368	0.007[a]
MC-NC	0.074	0.015[a]	0.019	0.377	0.384	0.144

[a] The Anosim and MRPP analyses of the three groups showed significant differences in the composition of the community structure between FMT and NC groups (Anosim analysis: p = 0.006; MRPP analysis: p = 0.007). Anosim analyses showed significant differences between MC and NC groups (p = 0.015).

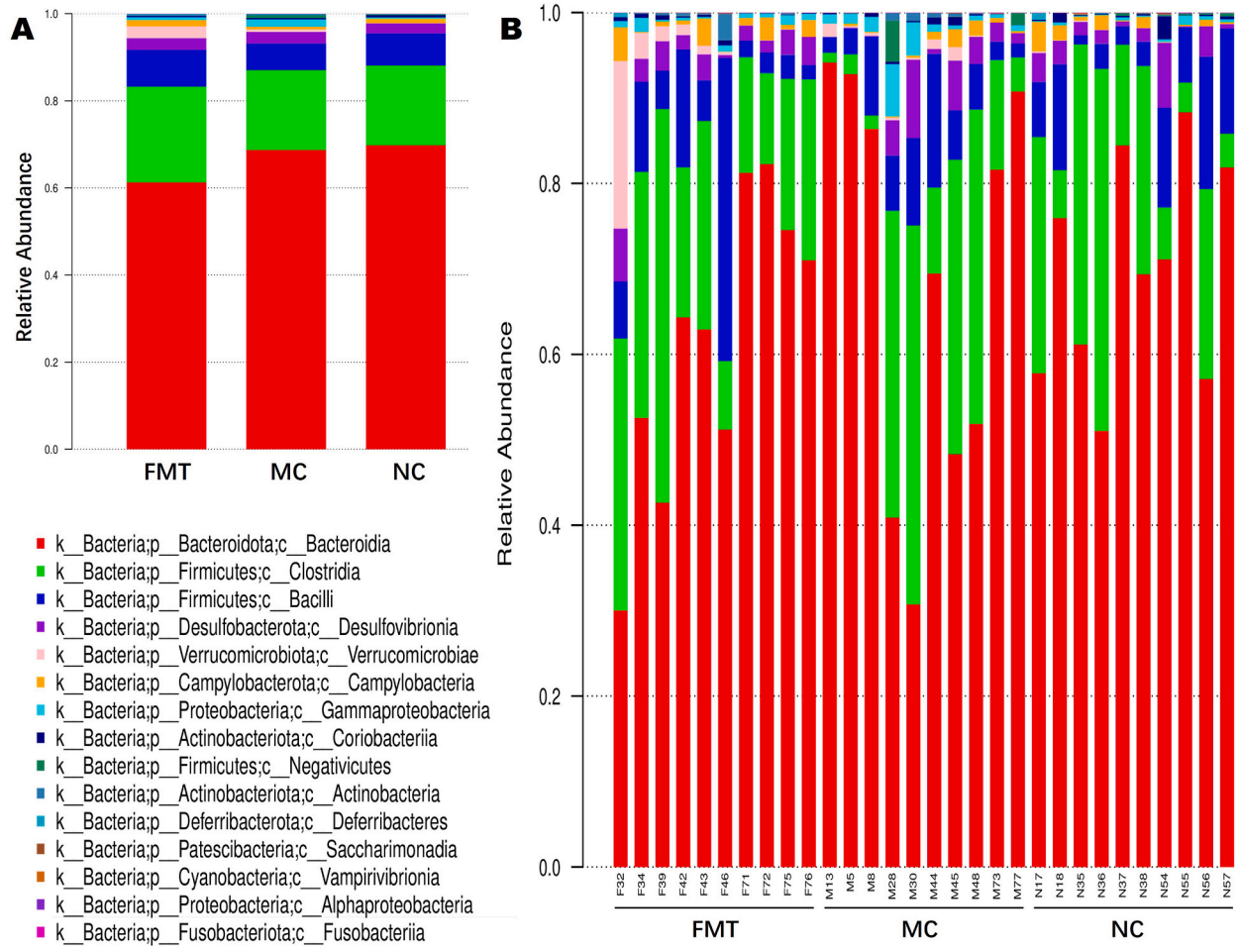

Fig. 3. Analysis of intestinal flora species abundance.
A: Bar chart of relative abundance of intestinal microflora of mice in each group (class level);B: Bar chart of relative abundance of intestinal microflora of mice in each sample (class level). The relative distribution of species at each level was demonstrated using R software. In the distribution of intestinal bacterial gates, compared to the NC and MC group, the abundance of Bacteroidetes exhibited a lower trend in FMT group, while Verrucomicrobia and Firmicutes were higher trend, with Verrucomicrobia being particularly increased. Compared to NC, the abundance of Proteobacteria was showed a higher trend in MC group, while Firmicutes was lower trend.

In order to understand the correlation between the relative abundance of differentially rich intestinal flora and the severity of EAE in the FMT group. A spearman rank correlation analysis was performed between neurological scores and 11 bacterial groups from FMT group mice, including p_Verrucomicrobiota, c_Verrucomicrobiae, o_Verrucomicrobiales, f_Akkermansiaceae, g_Akkermansia, s_Akkermansia_muciniphila, f_Bacteroidaceae, g_Bacteroides, s_Bacteroides_acidifaciens, g_Muribaculaceae, f_Muribaculaceae s_uncultured_organism. The results showed that the abundance of Verrucomicrobiota and the A. muciniphila of Verrucomicrobiota in each classification showed strong positive correlation with neurological score, with the correlation coefficients all equal to 0.569; the

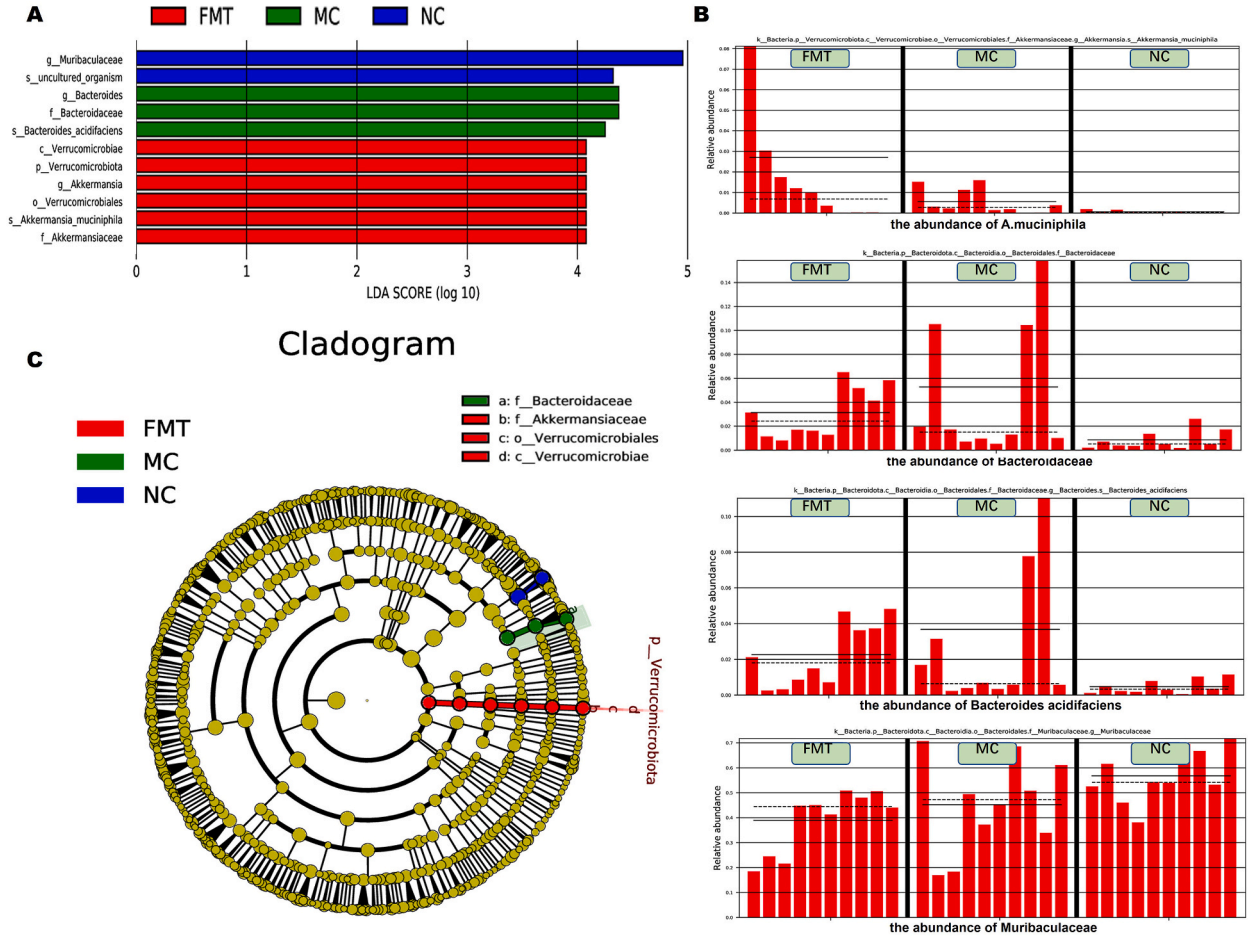

Fig. 4. Analysis of species differences between groups.

A-C: The abundance of A. muciniphila was significantly higher in the FMT group compared to MC while Bacteroidaceae and Bacteroides acidifaciens were lower in the FMT group. Muribaculaceae were more abundant in NC compared to MC and FMT (The intestinal flora profiles of the three groups were analyzed using LefSe.). A: The abundance of different species in the various groups. For the species with statistically significant differences between groups, whose LDA score is greater than the set value (default is 2). The length of the bar chart represents the impact size of the different species. B: According to the LefSe analysis, the bar chart of the relative abundance of four bacteria: A. muciniphila, Bacteroidaceae, Bacteroides acidifaciens, and Muribaculaceae. The horizontal axis shows the different groups of samples while the vertical axis shows relative abundance of species, the solid line represents the average relative abundance of all species in the group, the dotted line indicates the median of relative species abundance for all samples in the group. C: Evolutionary branching of intestinal flora with rich differences. The circles radiating from the inside to the outside represent taxonomic levels from phylum to genus (or species). Each small circle at different taxonomic levels represents a taxon at that level, and the diameter of the small circle is proportional to the relative abundance. Species without significant differences were uniformly colored yellow, and the colors of different species were the same as those of groups. Each colored node represents the microorganism group that plays an important role in the group.

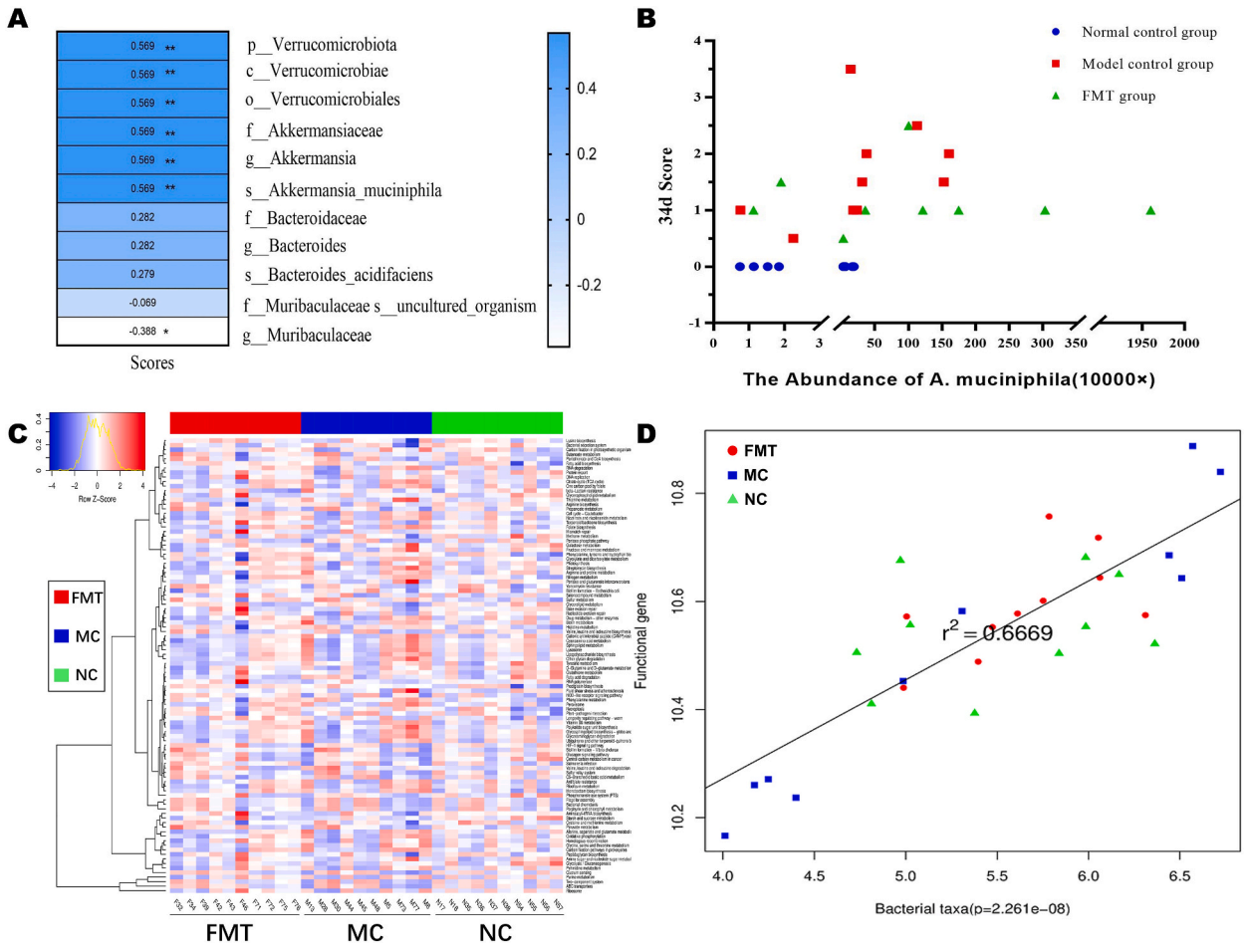

Fig. 5. Functional abundance profiles and predictive analysis of species and functional consistency.
A: Correlation analysis of species relative abundance and the neurological scores of EAE in the FMT group. The abundance of Verrucomicrobiota and the A. muciniphila of Verrucomicrobiota in each classification showed strong positive correlation with neurological score, with the correlation coefficients all equal to 0.569; the abundance of Muribaculaceae showed a negative correlation with neurological score, with the correlation coefficient of −0.388; Bacteroidaceae and Bacteroides acidifaciens had no significant correlation with neurological scores. (*$p < 0.05$, **$p < 0.01$) B: Scatter plot of of the correlation between the abundance of A. muciniphila and neurological scores of EAE at day34. C: Clustering diagram of the third level of functional abundance. The heatmap was generated by R software, and the method hclust was used to perform cluster analysis. Rows correspond to functions and columns to samples. The darker the blue, the lower the relative abundance, and the darker the red, the higher the relative abundance. D: Linear correlation analysis showed a strong positive correlation between intestinal flora species and function with a diversity correlation coefficient of r2 = 0.6669. The greater the r-squared value, the better the linear relationship between species and function, and the higher the consistency between species and function. ($0 < r < 1$).

abundance of Muribaculaceae showed a negative correlation with neurological score, with the correlation coefficient of −0.388; Bacteroidaceae and Bacteroides_acidifaciens had no significant correlation with that (Fig. 5A and B). It indicates that A. muciniphila is correlated with the symptoms of EAE after FMT treatment. We speculate that FMT treatment promotes the colonization of A. muciniphila in the gut of EAE.

⑤ Functional abundance profiles and predictive analysis of species and functional consistency

To further elaborate the correlation between the mouse intestinal flora and the alterations in intestinal microbial metabolic pathways, we conducted a correlation analysis between A. muciniphila and the biometabolic pathways. KEGG mainly divides biological metabolic pathways into three levels, and the third level is the pathway of KEGG (Fig. 5C), indicating that the metabolic pathways of mouse intestinal microbes were altered by FMT treatment. This showed a positive correlation between A. muciniphila and changes in 12 biometabolic pathways as shown in Table 3. These results suggest that FMT can alter the microbial metabolic environment of the mouse intestine by modulating the intestinal flora, which in turn attenuates EAE.

The diversity of species and function was calculated separately based on the relative abundance of species and function in faecal samples, and linear correlation analysis was performed. We found a strong positive correlation between intestinal flora species and

Table 3
Correlation analysis between *A. muciniphila* and biological metabolic pathways.

Biological metabolic pathways	*A. muciniphila*
Propanoate metabolism	0.451[a]
Pantothenate and CoA biosynthesis	0.438[a]
Quorumsensing	0.434[a]
Pyruvate metabolism	0.433[a]
ABC transporters	0.422[a]
Valineleucine and isoleucine biosynthesis	0.408[a]
Butanoate metabolism	0.406[a]
Valineleucine and isoleucine degradation	0.397[a]
Vancomycin resistance	0.395[a]
HIF1signaling pathway	0.385[a]
Two-component system	0.384[a]
Sulfurrelay system	0.369[a]

[a] Represents the correlation between the biological metabolic pathway and the abundance of *A. macuciniphila*. Higher values indicate stronger correlations ($p < 0.05$).

function with a diversity correlation coefficient of $r^2 = 0.6669$ (Fig. 5D).

2.2. Part 2

2.2.1. The therapeutic effect of FMT is partly dependent on the HPA axis

In order to investigate the relationship between HPA axis and the pathogenesis of EAE, explore the effect of HPA axis destruction on the therapeutic effect of FMT, the EAE animals were preconditioned by adrenalectomy. The symptoms of the EAE rats peaked at 10–12 days, and the score was stable for 2 days before FMT (Fig. 6A). The enrollment rate of EAE rats was 75 %, which developed EAE symptoms successfully after adrenalectomy, with the HE and Luxol fast blue myelin staining results conformed to the histopathologic characteristic of EAE (Fig. 6B–E). There were no rats died during the experiment. We found that weight loss was greater in the MC group than in the FMT group, indicating that FMT treatment attenuated weight loss. Weight loss continued in the SC group, while weight increased in the FMT-S group, suggesting that FMT attenuated weight loss despite the disruption of the HPA axis (Fig. 6F).

Before FMT intervention, we also found more severe neurological scores in the SC group compared to the MC group ($p < 0.0001$) and higher in the FMT-S group compared to the FMT group ($p = 0.0001$), indicating that disruption of the HPA axis was involved in modulating these symptoms. After the FMT intervention, the neurological score changes were significantly greater in the FMT group than that in the MC, SC and FMT-S group (all the $p < 0.0001$), indicating that FMT had a therapeutic effect on EAE and the efficacy was partly dependent on the HPA axis (Fig. 6G–Table 4).

2.2.2. FMT lowered serum cortisol and IL-17, and increased TLR-2; these effects were reversed by blocking the HPA axis

In order to verify whether the adrenalectomy was success, and identify the function of HPA axis in each group, we measured serum cortisol by Elisa. Our results showed that cortisol levels were significantly lower in the SC and FMT-S groups, compared to the NC group ($p < 0.001$) and the FMT group ($p < 0.001$). This indicates that adrenalectomy reduced cortisol secretion and reversed the effects of FMT. Cortisol levels were significantly higher in the MC group compared to the NC group ($p = 0.011$) and significantly lower in the FMT group compared to the MC group ($p < 0.05$). These data show that cortisol was increased at the onset of EAE and that this increase was attenuated by FMT treatment (Fig. 6H).

To confirm whether the destruction of HPA axis affects the serum levels of IL-17 and TLR-2, and whether the therapeutic effect of FMT on IL-17 and TLR-2 depends on HPA axis. We found that IL-17 levels were significantly higher in the MC group compared to the NC group ($p < 0.0001$) and lower in the FMT group compared to the MC group ($p < 0.0001$), which were also higher in the FMT-S group compared to the FMT group ($p < 0.0001$). These data show that FMT treatment reduced serum IL-17 but that this was reversed by blocking the HPA axis (Fig. 6I).

The levels of TLR-2 were higher in the MC group compared to the NC group ($p < 0.0001$) and and showed further increase in the FMT group compared to the MC group ($p < 0.0001$). However, the FMT-S group exhibited lower TLR-2 levels compared to the FMT group ($p < 0.0001$). These data show that EAE is associated with elevated serum TLR-2, that FMT treatment also increases TLR-2, but that this latter increase is attenuated by blocking the HPA axis (Fig. 6J).

2.2.3. HPA axis disruption alters the species abundance and diversity of the intestinal flora

Alpha and beta diversity analyses showed a greater change in species abundance and diversity of the intestinal flora before and after the intervention in both the FMT-S and SC groups (Fig. 7 A-F). The Anosim index further confirms a significant difference in the intestinal flora before and after the intervention in the SC group ($p = 0.024$), while no significant differences were observed in the other groups (Fig. 8 AB). Notably, the phylum Bacteroidetes exhibited a higher abundance trend in both the FMT-S and SC group, while the phylum Firmicutes displayed a lower abundance trend (Fig. 8C). At the genus level, the FMT-S and SC group demonstrated a greater prevalence of the genus phascolarctobacterium (Fig. 8D). At the species level, Bacteroides acidifaciens were the predominant flora in the FMT-S group, followed by Bacteroides vulgatus, whereas no specific dominant flora was observed in the SC and MC groups (Fig. 8E).

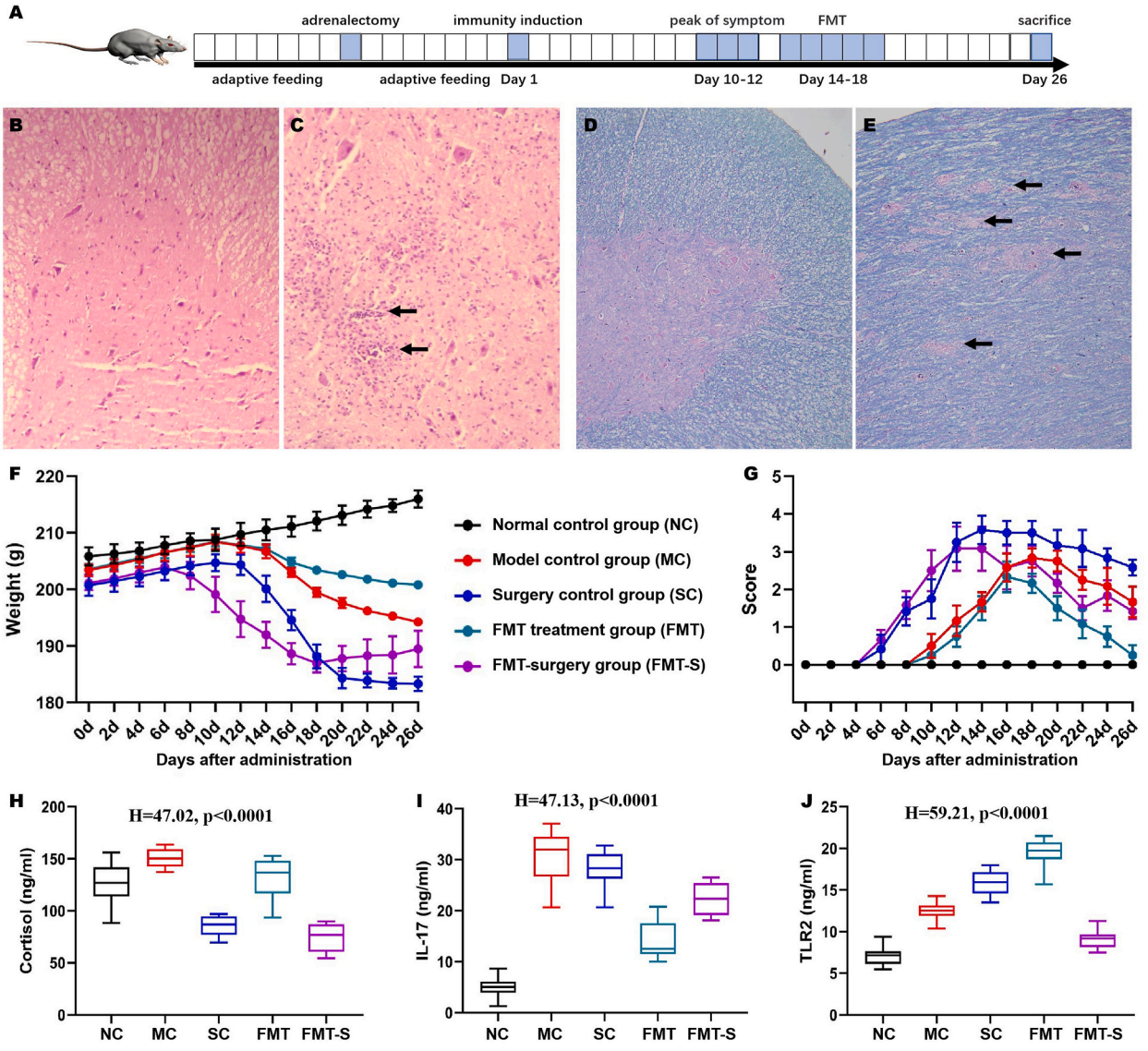

Fig. 6. The therapeutic effect of FMT is partly dependent on the HPA axis.
A: EAE rat experimental procedure. B–C: HE staining × 100. (B: normal spinal cord; C: EAE spinal cord, arrows indicate perivascular and parenchymal mononuclear cell infiltrates or cuffings). D-E: Luxol fast blue myelin staining × 100 (D: normal spinal cord; E: EAE spinal cord, arrows indicate demyelinations). F: Body weights in the different groups. The weight loss was greater in the MC group than in the FMT group, weight reduced more in the SC group, while increased in the FMT-S group. G: Neurological scores in the different groups. The general trend showed that more severe neurological scores in the SC group compared to the MC group and higher in the FMT-S group compared to the FMT group. H: Serum cortisol concentration of the five groups. The levels were significantly lower in the SC and FMT-S group, compared to the NC group (SC vs NC, $p < 0.0001$; FMT-S vs NC, $p < 0.0001$); which were significantly higher in the MC group compared to the NC group ($p = 0.0114$), and significantly lower in the FMT group compared to the MC group ($p = 0.043$) (Kruskal-Wallis non-parametric test, $H = 47.02$, $p < 0.0001$). I: Serum IL-17 concentration of the five groups. The levels were significantly higher in the MC group compared to the NC group ($p < 0.0001$), and lower in the FMT group compared to the MC group ($p < 0.0001$), which were also higher in the FMT-S group compared to the FMT group ($p < 0.0001$) (Kruskal-Wallis non-parametric test, $H = 47.13$, $p < 0.0001$). J: Serum TLR-2 concentration of the five groups. The levels were apparently higher in the MC group compared to the NC group ($p < 0.0001$), and increased more in the FMT group compared with the MC group ($p < 0.0001$), which were significantly lower in the FMT-S group compared to the FMT group ($p < 0.0001$) (Kruskal-Wallis non-parametric test, $H = 59.21$, $p < 0.0001$).

Table 4
Contrast of neurological scores in EAE before and after FMT treatment.

Group	n	Neurological scores		
		Before treatment	After treatment	Score changes
MC	6	2.83 ± 0.06	1.67 ± 0.41	1.16 ± 0.15[b]
SC	6	3.58 ± 0.38▲	2.59 ± 0.20[a]	0.99 ± 0.18[b]
FMT	6	2.23 ± 0.05▲★	0.23 ± 0.22Δ	2.00 ± 0.17
FMT-S	6	2.89 ± 0.27★◆	1.35 ± 0.26◊	1.44 ± 0.11[b]

Before treatment, compared to MC group, ▲$p < 0.05$ (SC vs MC, $p < 0.001$; FMT vs MC, $p = 0.0003$); compared to SC group, ★$p < 0.05$ (FMT vs SC, $p < 0.001$; FMT-S vs SC, $p < 0.001$); compared to FMT group, ◆$p < 0.05$ (FMT-S vs FMT, $p = 0.0001$) (one-way ANOVA followed by the LSD multiple-comparison test, $F = 71.06$, $p < 0.001$, degrees of freedom: within-group was 20, between-group was 3); Compared to pre-treatment, Δ$p < 0.05$ (paired samples *t*-test, degrees of freedom was 5, $t = 21.714$, $p < 0.001$); After treatment, compared to MC group.

[a] $p < 0.05$ (SC vs MC, $p < 0.001$); compared to FMT group, ◊$p < 0.05$ (FMT-S vs FMT, $p < 0.001$) (one-way ANOVA followed by the LSD multiple-comparison test, $F = 81.222$, $p < 0.001$, degrees of freedom: within-group was 20, between-group was 3); Score changes from before to after treatment, compared to FMT group.

[b] $p < 0.05$ (MS vs FMT, SC vs FMT, FMT-S vs FMT all the $p < 0.001$) (one-way ANOVA followed by the LSD multiple-comparison test, $F = 48.99$, $p < 0.001$, degrees of freedom: within-group was 20, between-group was 3).

3. Discussion

A growing number of studies support the important role of gut dysbiosis in MS[21]; rebuilding the gut flora may offer a novel and innovative approach to treating MS. Currently, FMT is considered to be the most direct method to reconstitute the intestinal flora[22]. However, how does FMT repair intestinal microecology to improve EAE and its exact underlying mechanisms of action remain unclear.

In our experiments, we found that EAE models with high neurological scores lost weight and were often incontinent, with loose stools or diarrhea. In contrast, FMT treatment attenuated this weight loss and resulted in less incontinence and improved EAE symptoms. Afterwards, we systematically examined the alteration of intestinal microecology by FMT and found that the EAE mice had a higher distribution of Bacteroidaceae, Bacteroides acidifaciens. The intestinal flora of EAE mice changed significantly after FMT treatment, with Verrucomicrobia, Akkermansia, and A. muciniphila species significantly increased in the FMT-treated mice. This indicates that the composition of the intestinal flora of EAE mice is characteristically altered after FMT treatment, and that these changes in flora may be associated with the pathogenesis of EAE. Therefore, key drivers of intestinal flora variability may be identified in future studies.

Notably, we also found that the intestinal flora of FMT-treated mice showed a significant increase in A. muciniphila at all taxonomic levels on the LDA score and LefSe. Thus, our results suggest that FMT treatment promotes the intestinal colonization of A. muciniphila in EAE mice. These bacteria are known to enhance the integrity of the intestinal epithelium and the thickness of the mucus layer, promoting intestinal health[23] and also play an important role in improving host metabolic function and immune responses [24].

It remains unclear whether A. muciniphila promotes disease onset and progression or protects the CNS. Several studies have shown that an increase in intestinal A. muciniphila is associated with the development of MS. Treatment of MS patients with probiotics was shown to promote a peripheral anti-inflammatory response and led to a reduction in intestinal A. muciniphila[25]. Increased levels of A. muciniphila have been found in the feces of MS patients, compared to the healthy population[26]. In addition, Li et al.[20] found that FMT treatment improved EAE symptoms and reduced intestinal A. muciniphila levels in mice. However, other studies suggest that intestinal A. muciniphila has CNS protective effects and can improve EAE symptoms. A. muciniphila was shown to ameliorate cognitive deficits in an Alzheimer's disease mouse model and showed protective effects against brain amyloid[27]. Oral administration of miR-30d from feces of MS patients was shown to suppress MS-like symptoms in mice by expanding A. muciniphila[28]. Gavage administration of norfloxacin to EAE mice delayed the symptoms of EAE by increasing the abundance of A. muciniphila, and inhibiting Th17 cell expression[29]. At present, the role of A. muciniphila and its underlying mechanisms in MS or EAE have not been fully investigated; its effect on the CNS needs to be further investigated. Although the results of these studies vary, the balance of data suggests that A. muciniphila may be a component of disease pathophysiology and a potentially important target for MS.

We also found that the relative abundance of A. muciniphila was positively correlated with the pyruvate fermentation metabolic pathway, indicating that FMT enriched A. muciniphila and its metabolic pathways in EAE mice. A similar study to ours found that transplantation of intestinal flora from MS patient donors into EAE mice increased the abundance of A. muciniphila in the pyruvate fermentation metabolic pathway, and that this change correlated with altered metabolic pathways in MS patients[20]. Although the impact of these metabolic pathways on the pathogenesis of EAE and MS is unclear, these changes suggest that A. muciniphila influence the development of EAE disease by modifying the metabolic environment in the mouse intestine by affecting pyruvate metabolism.

Meanwhile, we performed further experiments to test how disrupting the HPA axis affected the therapeutic effects of FMT. After bilateral removel of the rat adrenal glands, we observed a significant decrease in cortisol compared to the NC and FMT-S groups, confirming that the surgical removal of the adrenal gland was successful and that the HPA axis was disrupted. Cortisol levels were significantly higher in the MC group compared to the NC group, indicating activation of the HPA axis in the EAE pathogenic state. Compared with the MC group, the SC group showed significant weight loss and higher neurological scores, indicating that disruption of the HPA axis exacerbated the neurological deficits. In contrast, the FMT-treated rats showed less weight loss and lower neurological scores compared to the FMT-S group. Additionally, the changes in neurological scores before and after intervention were significantly smaller in the FMT-S group compared to the FMT group, indicating that blocking the HPA axis attenuated the therapeutic effect of FMT.

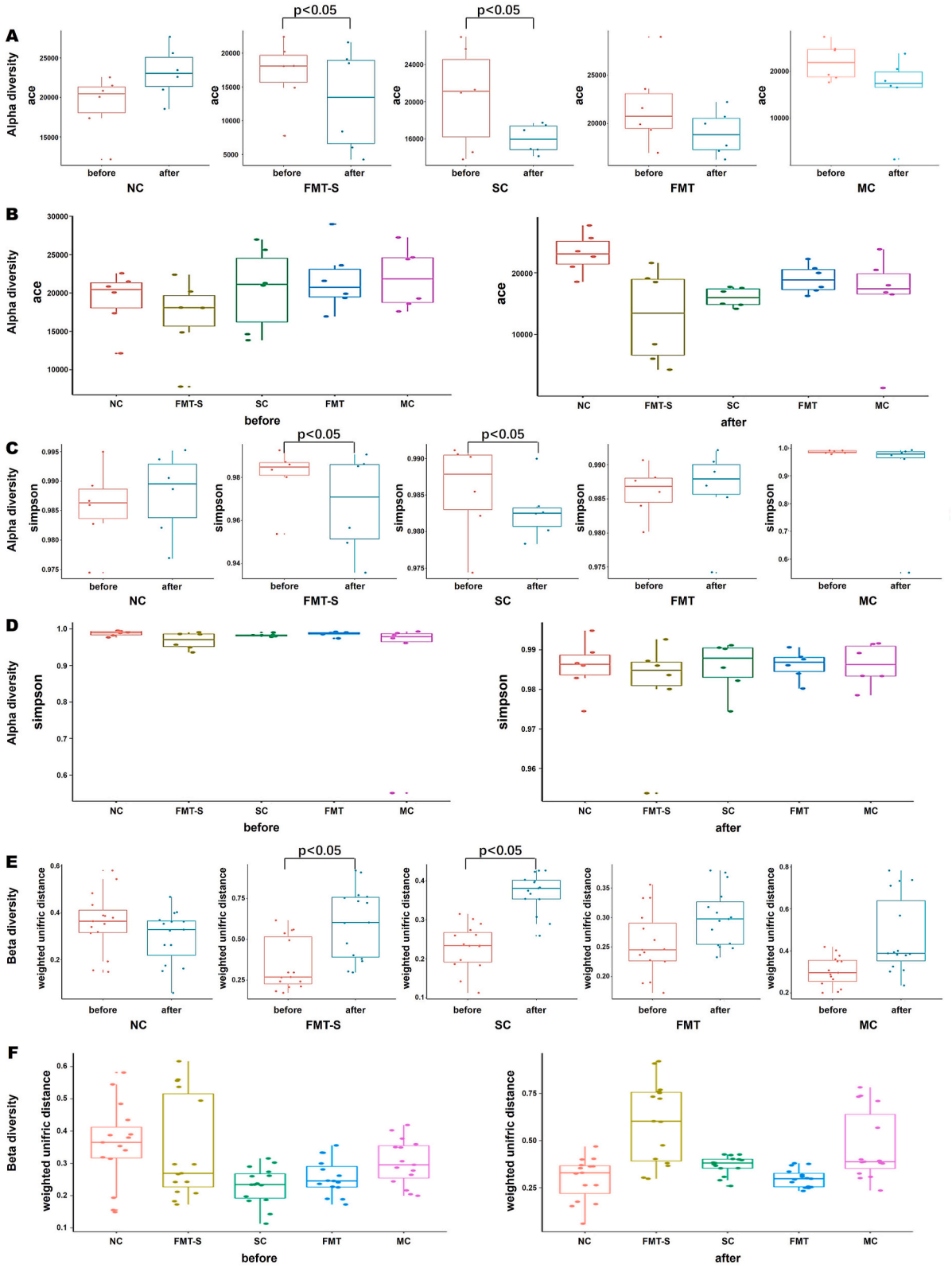

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Fig. 7. HPA axis disruption alters the diversity of the intestinal flora.
A. Within-group comparison of ace index before and after FMT treatment in each group. B. Between-group comparison of ace index in each group before and after FMT treatment. C. Within-group comparison of simpson index before and after FMT treatment in each group. D. Between-group comparison of simpson index in each group before and after FMT treatment. E. Within-group comparison of weighted unifrac distance before and after FMT treatment in each group. F. Between-group comparison of weighted unifrac distance in each group before and after FMT treatment. The larger ace index, the more species in the community; the larger simpson index, the higher diversity of the community. Alpha and Beta diversity analyses showed a greater change in species abundance and diversity of the intestinal flora before and after the intervention in the FMT-S group and the SC group.

We also found an increase in Bacteroidetes and a decrease in Firmicutes in both the FMT-S and SC groups. Bacteroidetes and Firmicutes account for approximately 98 % of the human intestinal flora, their relatively stable ratio maintains the balance of the intestinal flora and, when imbalanced, may lead to metabolic syndromes such as obesity and diabetes. Aho et al.[30] found a significant increase in the abundance of the phylum Bacteroidetes and a significant decrease in the abundance of the phylum Firmicutes in the gut of Parkinson's disease patients. Strength-trained EAE mice were found to show an increased abundance and diversity of the gut microbiota and a decrease of the Firmicutes/Bacteroidetes ratio (a significant increase in Bacteroidetes and depletion of Firmicutes), followed by a significant amelioration in symptoms of EAE[31]. Thus, the imbalance in the abundance of Bacteroides and Firmicutes may contribute to neuroimmune diseases.

In this study, we observed that Bacteroides acidifaciens was the dominant species in the FMT-S group. Interestingly, despite undergoing the same adrenalectomy procedure in both the SC and FMT-S groups, the SC group did not exhibit any specific dominant flora, suggesting that FMT treatment altered the composition of the gut microbiota and resulted in an increase in specific dominant flora.

We also found that serum IL-17 levels was higher in the EAE model compared to normal controls. These decreased after FMT treatment, but increased in the FMT-S group, indicating that blocking the HPA axis prevented the reduction in IL-17 by FMT. Studies have demonstrated that the progression of EAE disease is positively correlated with IL-17, and that IL-17-deficient mice are insensitive to EAE induction[32,33]. It has also been demonstrated that mice lacking IL-17A/F are resistant to EAE and that this is associated with an altered composition of the gut microbiota. IL-17A and IL-17F, instead of being encephalitogenic mediators, function as modulators of intestinal homeostasis, which in turn indirectly affects CNS-directed autoimmunity[34]. Therefore, IL-17 appears to be a key immunomodulatory mechanism of action underlying the effect of FMT on EAE.

We further delved into the effect of FMT on TLR-2. These innate immune receptors can mediate the inflammatory response associated with MS. Microbe-associated molecular patterns (MAMPs) in the circulatory system bind to TLRs and activate a series of intracellular responses that stimulate the release of pro-inflammatory cytokines, thereby triggering an inflammatory response. Patten et al.[35] found microglial proliferation and TLR-2 and TLR-4 mRNA upregulation in the brains of MS patients. Labib et al.[36] reported a significant increase in TLR-2 expression in lymphocytes and neutrophils of MS patients compared to healthy individuals, TLR-4 was significantly increased in lymphocytes. In our study, we found that serum TLR-2 was highly expressed at the onset state of EAE, consistent with previous clinical studies. However, surprisingly, we found that FMT treatment further increased TLR-2 levels. We hypothesize that FMT alters intestinal microecology, increasing gut microbial metabolites, which may increase MAMPs in the circulatory system, and finally trigger TLR activation. TLR-2 levels was significantly lower in the FMT-S group compared to the FMT group, indicating that blocking the HPA axis reversed the TLR-2 promoting effect of FMT.

Currently, the HPA axis is recognized as a key role in the pathogenesis of MS and a potential therapeutic target for MS[37]. A postmortem study showed that the HPA axis is activated in most MS patients and associated with MS progression and co-morbid mood disorders[38]. Germ-free mice lacking intestinal flora exhibit an increased HPA axis response to stress, which in turn exacerbates EAE [39]. These findings are similar to ours, which showed that surgical disruption of the HPA exacerbated EAE neurological deficits, and that blocking the HPA axis reversed the downregulation of serum IL-17 and the upregulation of serum TLR-2 by FMT. Nevertheless, we also found some differences in neurological scores, IL-17 and TLR-2 between the FMT-S group and the SC group, indicating that adrenal excision cannot block the overall efficacy of FMT treatment. Thus, the therapeutic effect of FMT on EAE may be partly dependent on the HPA axis.

In summary, FMT has a protective effect on EAE. FMT reduced weight loss and improved neurological impairments in EAE by reducing the levels of the pro-inflammatory cytokine IL-17, lowering serum cortisol, promoting serum TLR-2, increasing the abundance of beneficial flora A. muciniphila, and re-establishing intestinal homeostasis. Blocking the HPA axis reversed the effects of FMT in downregulating serum IL-17 and promoting serum TLR-2. The therapeutic effect of FMT on EAE is partly dependent on the HPA axis (see Fig. 9).

Statement of ethics

All the animal experiments were performed in accordance with institutional animal ethics guidelines, and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Guangdong Pharmaceutical University with the ethics approval number is SKJ2020003.

Data availability statement

The raw data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

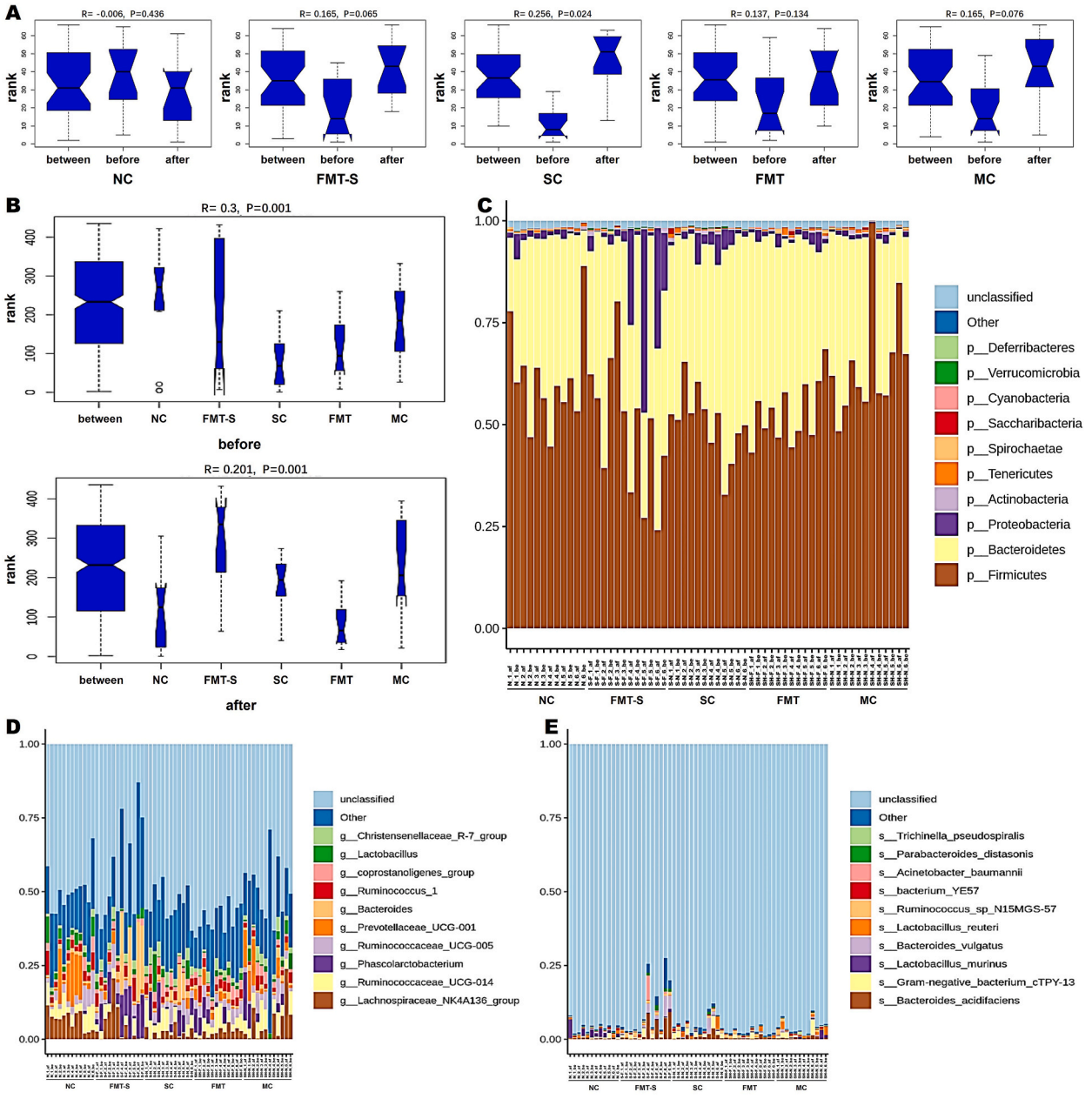

Fig. 8. HPA axis disruption alters the species abundance of the intestinal flora and promotes specific dominant flora.
A. The Anosim index in each group before and after FMT treatment (NC: R = 0.006, p = 0.436; FMT-S: R = 0.165, p = 0.065; SC: R = 0.256, p = 0.024; FMT: R = 0.137, p = 0.134; MC: R = 0.165, p = 0.076). B. Horizontal comparison of Anosim index in each group before and after FMT treatment (before: R = 0.3, p = 0.001; after: R = 0.201, p = 0.001). The Rank indicates the distance between pairs of samples, which was calculated by Mothur software based on unweighted unifrac distance. The Anosim index showed a significant difference in the intestinal flora before and after FMT treatment in the SC group, while no significant differences were observed in the other groups. C: Stacked map of species distribution at phylum taxonomic level. The Bacteroidetes exhibited a higher trend of abundance in both the FMT-S and SC group, while the Firmicutes displayed a lower trend of abundance. D: Stacked map of species distribution at genus taxonomic level. The FMT-S and SC group displayed a greater prevalence of the genus phascolarctobacterium. E: Stacked map of species distribution at species taxonomic level. Bacteroides acidifaciens were the predominant flora in the FMT-S group, followed by Bacteroides vulgatus, whereas no specific dominant flora was observed in the SC and MC group.

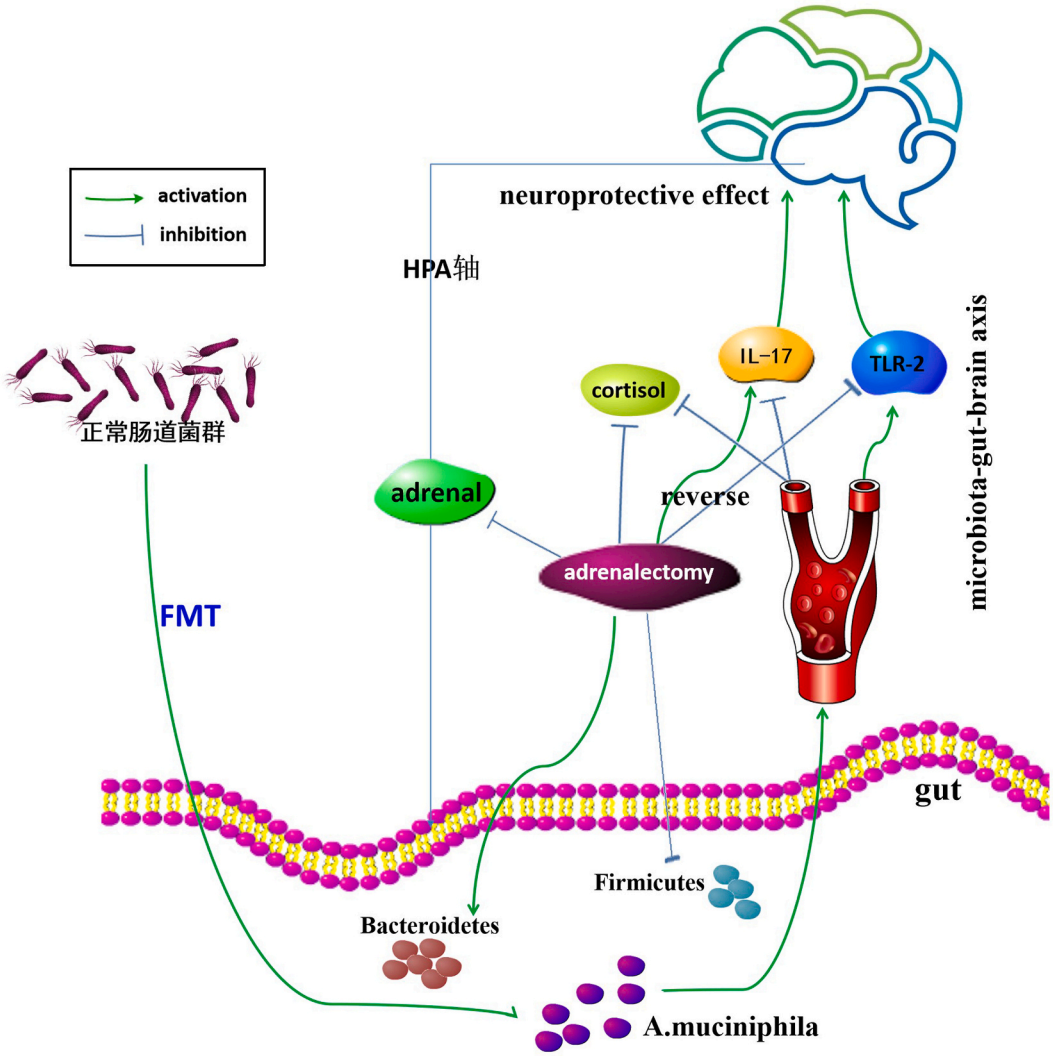

Fig. 9. Schematic illustration of the potential mechanisms of FMT on EAE.
FMT has neuroprotective effect on EAE by reducing the levels of the pro-inflammatory cytokine IL-17, lowering serum cortisol, promoting serum TLR-2, increasing the abundance of beneficial flora A. muciniphila. Blocking the HPA axis reversed the effects of FMT in downregulating serum IL-17 and promoting serum TLR-2, altered the abundance and diversity of the intestinal flora including an increase in Bacteroidetes and a decrease in Firmicutes.

CRediT authorship contribution statement

Danhong Xu: Writing – original draft, Project administration, Investigation. **Linxiang Ren:** Writing – original draft, Project administration, Investigation. **Wenbin Zhang:** Project administration, Investigation. **Shaohua Wu:** Formal analysis, Data curation. **Minling Yu:** Formal analysis, Data curation. **Xingxiang He:** Writing – review & editing, Supervision, Conceptualization. **Zhisheng Wei:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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