

Icariin Combined with Probiotics Ameliorates MPTP-Induced Parkinson's Disease in Mice via Gut Microbiota Regulation and the Ras Signaling Pathway

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Running title:

Icariin plus probiotics in PD mice

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Abstract:

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons and the aggregation of α -synuclein (α -syn), with limited effective treatment options available currently. This study investigated the therapeutic effect and mechanism of Icariin (ICA) combined with *Enterococcus faecium* R-026 and *Bacillus subtilis* R-179 on MPTP-induced PD in mice.

The results showed that the motor function of PD mice was significantly improved after combined drug treatment. Biochemical analysis indicated that in the combined treatment group, the activities of Superoxide dismutase (SOD) and Catalase (CAT) were increased, the content of Dopamine (DA) in brain tissue was elevated, the level of Malondialdehyde (MDA) was reduced, and the content of Glutathione (GSH) was restored simultaneously. Immunofluorescence staining results revealed that the relative number of Tyrosine hydroxylase (TH)-positive neurons in the brain tissue of mice in the MPTP group was significantly lower than that in the control (Con) group; meanwhile, the number of α -syn-positive neurons in the brain regions of mice in the MPTP group was significantly increased. At the molecular level, the combined treatment downregulated the expression of α -syn, Nuclear factor kappa-B (NF- κ B), Tumor necrosis factor- α (TNF- α), Interleukin-6 (IL-6), and BCL2-Associated X (BAX), while upregulating the expression of TH, B-cell lymphoma/leukemia-2 (Bcl-2), PTEN-induced kinase 1 (PINK1), and Inhibitor of nuclear factor

kappa B alpha ($I\kappa B\alpha$). Gut microbiota analysis demonstrated that the combined treatment reversed dysbiosis and restored the abundances of Akkermansia and Lactobacillus. Transcriptomic analysis showed that the Ras signaling pathway was activated, and the expressions of the Epha2, Angpt2, Tgfa, and pla2g4a genes were upregulated.

These findings suggest that Icariin combined with probiotics exerts a significant therapeutic effect on MPTP-induced PD in mice, and its action may be mediated by antioxidant activity, gut microbiota regulation, and Ras signaling pathway modulation. This provides experimental evidence for its potential application in the management of Parkinson's disease.

Keywords: Probiotics; Parkinson's disease; Gut microbiota; Oxidative stress; 16S rDNA

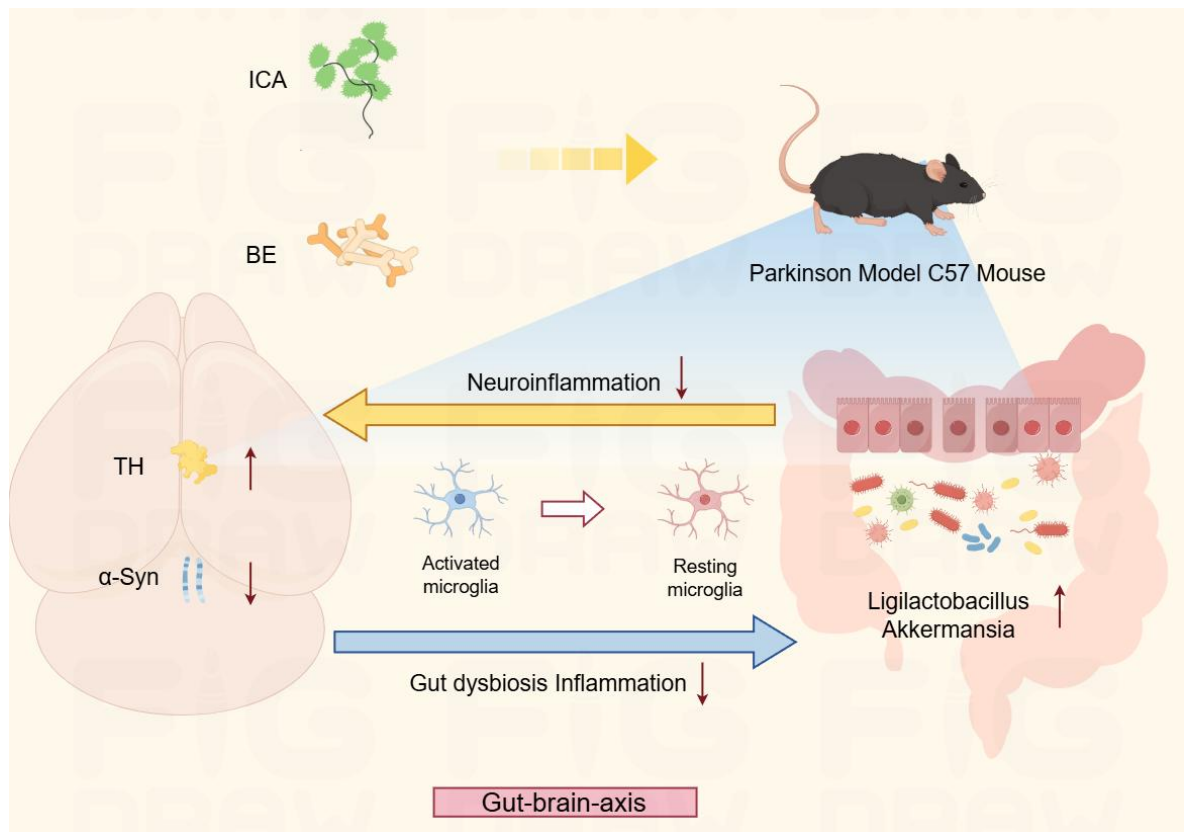


Figure 1 The gut-brain axis (GBA) is a crucial molecular communication network between the microbiota and the brain. This study investigated the therapeutic effect and mechanism of ICA combined with *Enterococcus faecium* R-026 and *Bacillus subtilis* R-179 on MPTP-induced PD mice. Abbreviations: PD: Parkinson's disease, ICA: Icariin, BE: *Enterococcus faecium* R-026 and *Bacillus subtilis* R-179, TH: Tyrosine hydroxylase.

Statements and Declarations

Competing Interests

The authors declare no competing interests, financial or otherwise.

Funding

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Ethics Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Beihua University (Approval No. 2024082117) and performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Author Contributions

Xiao Guo and Wanqiao Wu contributed equally to this work. Liping An conceived and designed the study. Xiao Guo, Wanqiao Wu, Qiaoran Liu, Jiaying Xing, Zhenzhen Wu, Xinlin Shi, and Yuhong Zhang performed the experiments and analyzed the data. Xiao Guo and Liping An wrote the manuscript. All authors read and approved the final manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

1 Introduction

Parkinson's Disease (PD) is a common neurodegenerative disease, and it is the second most common neurodegenerative disease after Alzheimer's disease. It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra - striatum region and a significant reduction in the content of dopamine in the striatum. Nearly 1% of the global population over 60 years old suffers from Parkinson's disease which seriously affects the quality of life of patients^[1]. With the serious aging of the population, the prevalence rate of PD is showing a significant upward trend.

As a microbial preparation, probiotics have long been used to improve human health. Regulation of intestinal flora can be achieved through dietary supplements or probiotic intake. Studies have shown that intake of certain specific probiotics can effectively prevent and improve PD through the gut-brain axis, with potential effects including regulating intestinal microecology, reducing inflammatory responses, and promoting the expression of neurotrophic factors^{[2][3]}. It has been found to delay aging and prevent neuro degenerative diseases^[4]. After transplanting *Enterococcus faecium* into PD mice, the dopamine level in the mice's brains was significantly increased, and the PD symptoms were improved^[5]. *Bacillus subtilis* has been proven to restore gastrointestinal dysbiosis and has good tolerance in the gastrointestinal tract^[6]. It can effectively combat the accumulation of α -Syn and even clear some accumulated α -Syn, thereby delaying the progression of PD^[7]. Recently, studies have found that *Lactobacillus plantarum* DP189 is an effective psychobiotic, which can reduce α -synuclein (α -SYN) aggregation in MPTP-induced Parkinson's disease (PD) mice by regulating oxidative damage, inflammation, and gut microbiota dysfunction^[8].

Epimedium is a genuine Chinese herbal medicine in Guizhou with a long history of application. It belongs to the Berberidaceae family and was first recorded in Shennong's Classic of Materia Medica. Also known as "Xianlingpi", it has many species and a wide distribution. Epimedium has the effects of

tonifying the kidney and strengthening yang, dispelling wind and dampness, and strengthening muscles and bones. It contains many monomers, mainly flavonoids, among which icariin has the highest content but a low metabolic rate. Studies have found that icariin (ICA) has various pharmacological effects such as neuron protection, anti-inflammation, anti-oxidative stress, and bone protection [9][10]. ICA plays a protective role in neurological diseases such as PD^[11], and Alzheimer's disease (AD) [12]. It can improve dopamine (DA) in the striatum of PD mice and reduce the loss of dopaminergic neurons in the substantia nigra [13].

In this study, we investigated the therapeutic effects of ICA combined with *Enterococcus faecium* and *Bacillus subtilis* in an MPTP-induced PD mouse model. The treatment efficacy was systematically evaluated, and potential mechanisms were preliminarily explored through gut metagenomics and transcriptomics analyses. This research aims to provide reliable evidence for the combined application of traditional Chinese medicine and microbial therapy in preventing and treating PD and other neurodegenerative disorders.

2 Materials and methods

2.1 Experimental Animal

C57BL/6J mice (bodyweight: (20 ± 2) g) were purchased from Liaoning Changsheng biotechnology co., Ltd (Shenyang, China). Laboratory animal production license number: SCXK 2020-0001. Ethical approval number: 2024082117.

2.2. Chemicals and materials

Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was purchased from Shanghai Yuanye Bio-Tech Co., Ltd. Icariin was purchased from Nanjing Zelang (purity, 98%) Bio-Tech Co., Ltd. L-Dopa was purchased from Sigma. *B. subtilis* R179 and *E. faecium* R-026 strains were obtained from a commercial probiotic product made by Medilac-S (Beijing Hanmi Pharmaceutical Co. Ltd., China). Malonaldehyde (MDA), superoxide dismutase (SOD), Glutathione (GSH) assay kits and Dopamine (DA) ELISA kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RNAiso Reagent, cDNA synthesis Kit and RT-PCR Kit were purchased from Takara Bio, Inc. Marker and Enhanced chemiluminescence (ECL) detection kit were purchased from Sevier Biological technology Co. Ltd. Rabbit anti-mouse α -synuclein antibodies, tyrosine hydroxylase (TH), dopamine transporter (DAT), nuclear factor- κ B (NF- κ B), nuclear factor-kappa B inhibitor alpha (I κ B α), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), Bcl-2-associated X

protein (BAX), B-cell lymphoma-2 (BCL-2), PTEN-induced kinase 1 (PINK1) and the corresponding secondary antibodies were all purchased from Diagbio Co., Ltd. All of the other reagents with a high analytical purity grade were purchased from Dingguo Changsheng Biotechnology Co., Ltd. The primers were synthesized by Sangon Biotech company.

2.3 Animals and experimental design

The mice were divided into six groups (n=10 per group). Control group (Con), Model group (MPTP), Icaritin treatment group (ICA), Probiotics treatment group (BE), Combination treatment group (C) and Positive drug group (Pos). Except for the Con group, all other groups received intraperitoneal (i.p.) injections of MPTP at a dose of 30 mg/kg daily for 7 consecutive days. The Con group was administered an equivalent volume of saline instead^[14].

ICA group: after modeling, ICA were given by gavages according to 50 mg/kg doses as single administration for 14 days. BE group: mice were orally immunized by gavage with subtilis R179 (1×10^7) and E. faecium R-026 (0.9×10^8) FFU for 14 days. C group: ICA (50 mg/kg) was administered intragastrically and probiotics administration method was the same as described above.

2.4 Behavioral Experiments

In the pole test, mice were situated on the top of a vertical wooden pole, 50 cm long and 1 cm in diameter, with a rough texture, and they autonomously descended^[15].

In the swim experiment, the mice were carefully placed in the water and permitted to swim freely for 2 minutes. The scoring criteria for the swimming test were shown in Table 1^[16].

In the Open Field Test, mice were placed in individual open-field chambers, and their behavior was tracked for 5 minutes. Each of the above experiments was carried out three times independently and repeated^[17].

2.5 Biochemical Indicators Analysis

After the behavioral tests, mice were sacrificed, and the blood was collected for serum separation. Mouse brain tissue were collected from each group and lysed in total lysis buffer. The activity of CAT, and SOD, and content of MDA, GSH and DA were determined following the manufacturer's recommendations.

2.6 HE Staining

Biopsy specimens were immersed in 10% formalin, embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin according to routine protocols. Finally, they were

dehydrated with graded alcohol and rendered transparent by xylene liquid, and coverslipped with a permanent mounting medium. The stained sections were observed under a microscope.

2.7 Immunofluorescence Staining of Brain Tissue

Mouse brain tissues were harvested and fixed overnight in 4% paraformaldehyde. After fixation, the brain tissues were transferred to 30% sucrose solution at 4°C for dehydration for 3 days. Once fully dehydrated, the brain tissues were taken out, blotted dry of surface liquid with absorbent paper, embedded in embedding medium, quickly frozen in liquid nitrogen, and sectioned using a cryostat. The brain sections were immersed in PBS buffer containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for blocking for 1 hour. After blocking, the sections were rinsed three times with BBS buffer, then incubated overnight at 4°C with primary antibodies diluted at a ratio of 1:500 in 0.1% PBST solution. Following primary antibody incubation, the sections were rinsed three times with PBS, then incubated with secondary antibodies for 1 hour. After secondary antibody incubation, the sections were incubated with DAPI for 5 minutes, and finally rinsed three times with PBS.

2.8 16s rRNA sequencing analysis

Stool samples were collected in sterile tubes and stored at -80°C until further analysis. The DNA extraction and sequencing were performed by the Hangzhou Lianchuan Biotechnology Company. For sequencing, the selected V3-V4 variable region was amplified with polymerase chain reaction (PCR) using high-fidelity DNA polymerase and primers with specific barcodes. The constructed library was then sequenced with Illumina platforms. Species annotation analysis was executed on the OTUs representative sequence with the help of the Mothur method and the SILVA SSUrRNA database. In addition, alpha diversity and beta diversity analyses, along with correlation and functional prediction analyses, can demonstrate the variations between samples.

2.9 Transcriptomic Analysis of the Substantia Nigra in Mouse Brain Tissue

Mice in each group were sacrificed to collect substantia nigra brain tissue. For RNA analysis, the tissues were always immersed in RNAlater stabilization reagents to prevent RNA degradation. Total RNA was extracted using Trizol RNA extraction kit. RNA concentration, purity, and integrity was assessed by NanoDrop and Agilent Bioanalyzer. The qualified RNA samples were used for cDNA library construction. And the optimal sample size was selected and sequenced using an Illumina sequencing platform. Transcriptomic analysis of the mouse brain tissue was performed by

Hangzhou Lianchuan Biotechnology Company. Gene expression levels were quantified using RSEM software. Differential expression analysis was performed using tools DESeq2 and EdgeR. To explore gene function and the regulatory networks involved in the mice brain, GO function and KEGG pathway enrichment networks were performed to analyze genes on the Metascape website.

2.10 Quantitative Real-Time PCR

Total RNA was extracted using RNAiso Reagent and quantified using NanoDrop One spectrophotometer. Then, RNA was reverse-transcribed to cDNA using All-in-One cDNA Synthesis SuperMix. Real-time quantitative RT-PCR (qRT-PCR) was conducted with ChamQ Universal SYBR qPCR Master Mix. The 2- $\Delta\Delta C_t$ method was used to calculate relative gene expression, with GAPDH used as a reference gene. The primer sequences are presented in Table 1.

Table 1 Primer pairs for genes detected by real-time PCR

Genes	primer sequences (5'-3')	
Epha2	Forward	GCACAGGGAAAGGAAGTTGTT
	Reverse	CATGTAGATAGGCATGTCGTCC
Angpt2	Forward	AACTCGCTCCTTCAGAAGCAGC
	Reverse	TTCCGCACAGTCTCTGAAGGTG
Tgfa	Forward	CTCTGGAGAACAGCACATCC
	Reverse	CACAGGTGATAATGAGGACA
pla2g4a	Forward	AAAAGAGTACCAAAGCGACAAC
	Reverse	CGTCCTTCTCGGGTATTGAATA
GADPH	Forward	GCACAGTCAAGGCCGAGAAT
	Reverse	TGGTGAAGACACCAGTAGA

3 Experimental Results

3.1 Behavioral results

For a comprehensive assessment of the intervention efficacy of ICA in combination with BE in PD mice, we measured motor coordination by analyzing the time required for pole-climbing behavior. Hanging test showed significantly decreased in the MPTP group compared to Con group ($p < 0.01$). Compared with the MPTP group, the score of the C group was notably higher ($p < 0.01$). In the swimming test, the scores of treatment groups were significantly higher than those of MPTP group ($p < 0.05$). Schematic of the single field test results showed that compared with MPTP

group, C group demonstrated both significantly higher movement speed and considerably greater travel distance ($p<0.01$). The results of the above behavioral tests showed that ICA alone ameliorated behavioral symptoms in PD mice, the adjunctive probiotic treatment yielded significantly enhanced therapeutic outcomes.

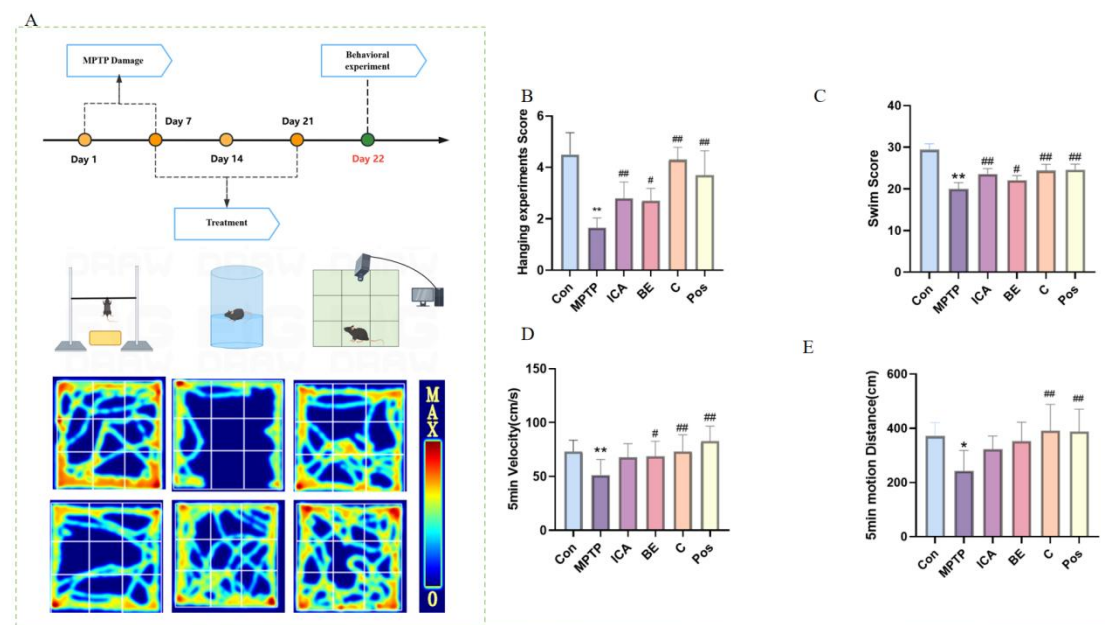


Figure 2 (A) Behavioral results (B) Results of pole climbing test (C) Results of swimming test (D) Average speed within 5 minutes (E) Total movement distance within 5 minutes Note: Compared with the Con group, * $p<0.05$, ** $p<0.01$; compared with the MPTP group, # $p<0.05$, ## $p<0.01$.

3.2 Determination results of SOD, MDA, GSH, and CAT in mouse serum, and DA in brain tissue

We investigated the effects of drug treatment on oxidative stress status and neuroprotective effects in PD mice. The serum and brain tissue biochemical indexes of mice are shown in Figure 3. The results demonstrated that compared with Con group, MPTP-treated mice showed significantly decreased serum activities of SOD ($p<0.01$) and CAT ($p<0.05$), along with significantly increased MDA ($p<0.01$) levels, indicating enhanced oxidative stress. Furthermore, the MPTP group exhibited a marked reduction in DA ($p<0.01$) content in brain tissues, suggesting impairment of dopaminergic neurons. After treatment with ICA, BE, their combination, and the positive control drug, mice showed significant restoration of serum SOD ($p<0.01$) and CAT activities and a notable decrease in MDA ($p<0.01$) levels. Additionally, DA ($p<0.01$) content in brain tissue partially recovered. These results suggest that these interventions offer neuroprotection by reducing oxidative stress and preserving dopaminergic neurons, providing valuable insights into PD pathogenesis and

potential treatments.

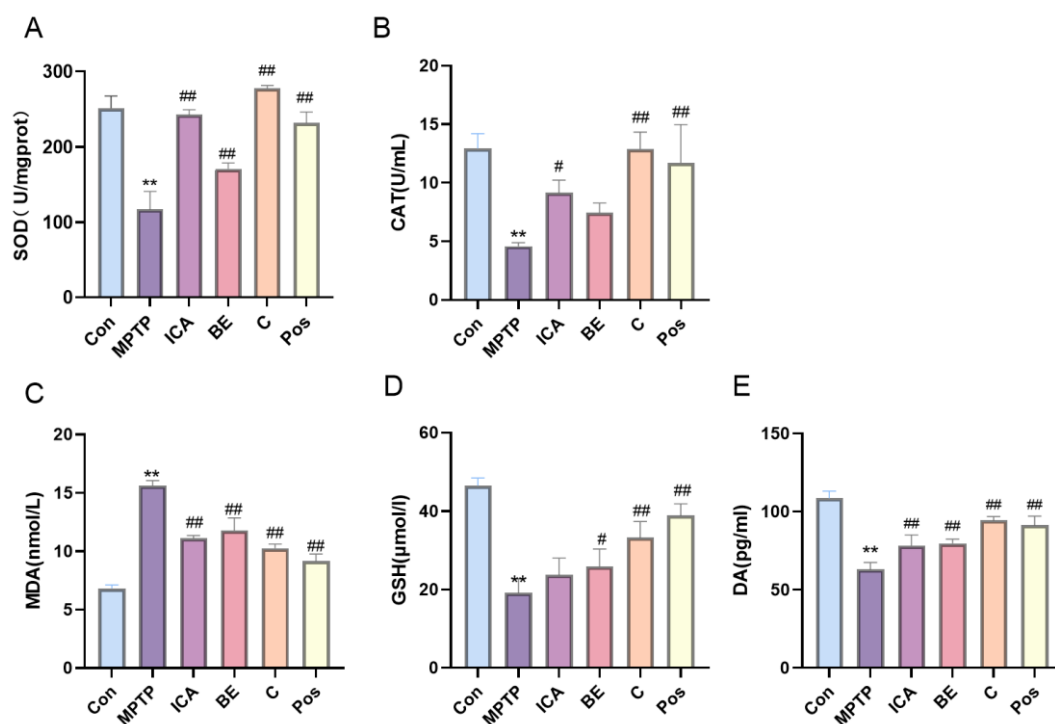


Figure 3 Determination results of SOD, MDA, GSH, CAT in mouse serum and DA in brain tissue (A) SOD (B) CAT (C) MDA (D)GSH (E)DA

3.3 HE staining results

The results of HE staining revealed that in the Con group, striatal cells exhibited a well-organized arrangement with normal neuronal morphology. The nuclei were clearly visible, centrally located with prominent nucleoli, and the cytoplasm displayed uniform staining without any indications of cellular edema or degeneration (Figure A). Conversely, the MPTP group exhibited significant pathological alterations in the striatum, including a marked reduction in neuronal density, disorganized cellular arrangement, shrunken neuronal soma with occasional vacuolar degeneration, and evident interstitial edema (Figure B), indicative of degenerative damage to dopaminergic neurons. All treatment groups showed varying degrees of improvement in striatal pathology compared to the MPTP group. Notably, the C group and Pos group demonstrated the most significant improvements, with increased neuronal density relative to the MPTP group and more regular cellular organization (Figures E, F). These results suggest that the combination therapy significantly alleviates MPTP-induced morphological damage to striatal neurons in PD mice, maintaining cellular structural integrity. The neuroprotective efficacy was comparable to that of the positive drug, potentially mediated through inhibition of inflammatory responses or oxidative stress pathways.

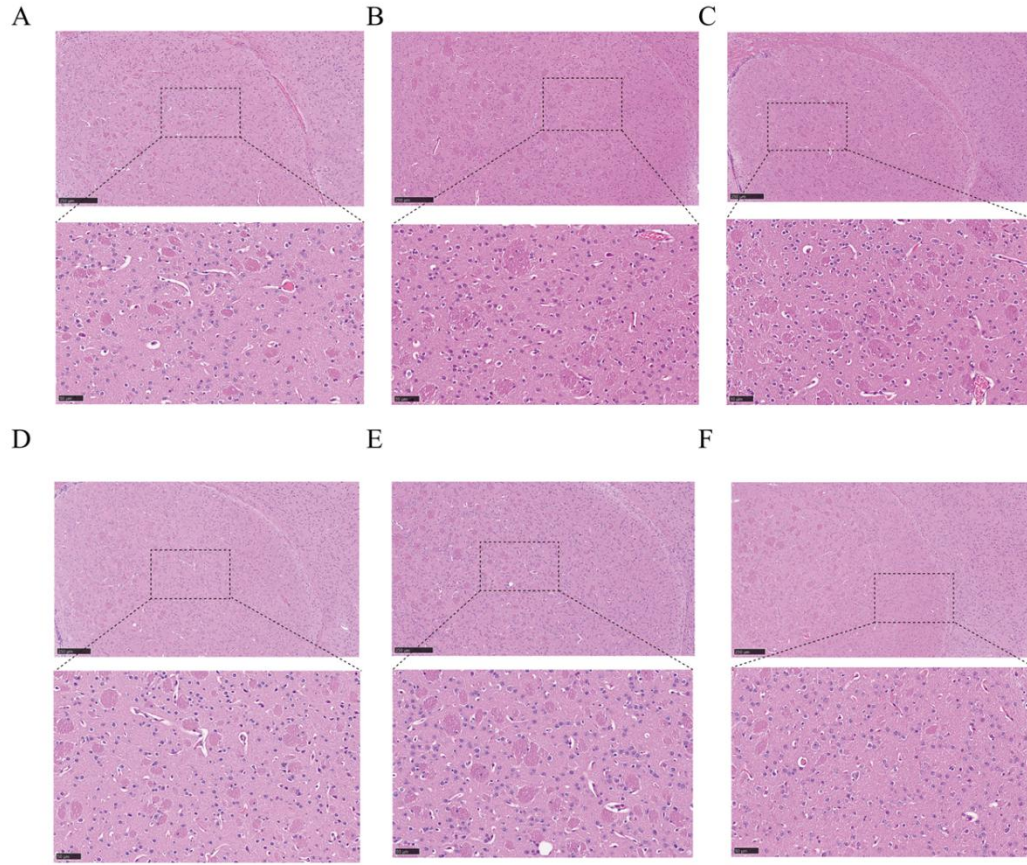


Figure 4 HE stain (A)Con (B)MPTP (C) ICA (D)BE (E)C (F)Pos

3.4 Immunofluorescence Staining of Brain Tissue

The results of immunofluorescence staining showed that the relative number of TH-positive neurons in the brain tissue of mice in the MPTP group was significantly lower than that in the control group (Con). This is highly consistent with the pathological feature of massive loss of dopaminergic neurons in the substantia nigra of clinical PD patients, which further confirms the pathological reliability of the MPTP-induced PD model. Meanwhile, the number of α -syn-positive neurons in the brain region of mice in the MPTP group was significantly increased — abnormal aggregation of α -syn is a typical pathological marker of PD. However, the combined treatment with icariin and probiotics could effectively alleviate this abnormal increase, suggesting that the combined intervention may exert neuroprotective effects by protecting dopaminergic neurons and inhibiting the abnormal accumulation of α -syn.

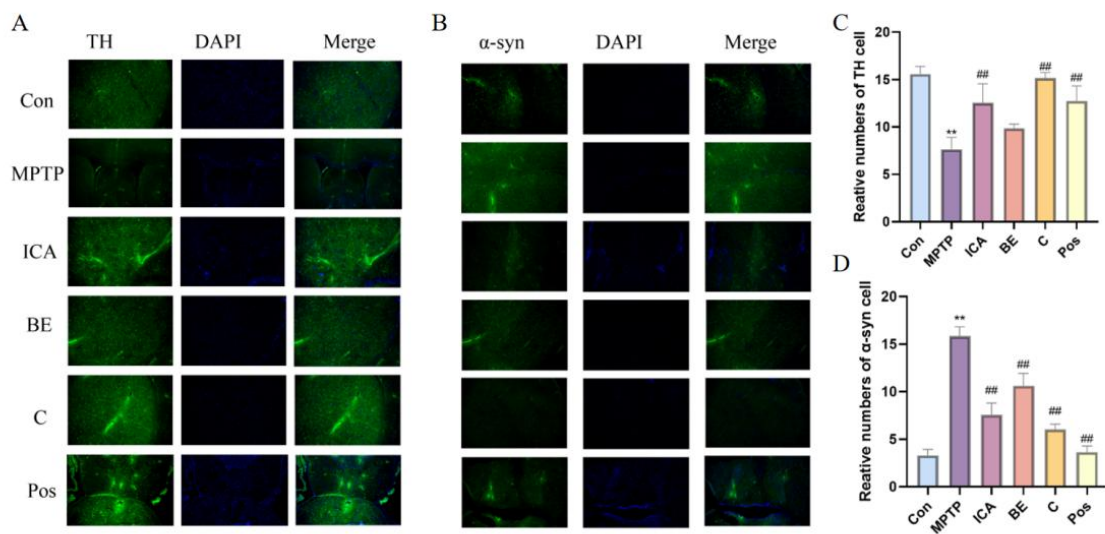


Figure 5 Immunofluorescence Staining (A) Representative immunofluorescence images of TH-positive neurons (B) Representative immunofluorescence images of α -syn-positive neurons (C) Comparison of the relative number of TH neurons among different groups (D) Comparison of the relative number of α -syn neurons among different groups

3.5 Detection of protein expression in mouse brain tissue by Western blot

In this study, Western blot analysis was used to detect the expression levels of various proteins in the brain tissues of mice with Parkinson's disease. The expression level of TH in the MPTP group was significantly decreased ($P < 0.01$), which indicated that MPTP induction could cause damage to the function of dopaminergic neurons; while the expression of TH protein in group C was significantly increased ($P < 0.01$), suggesting that the combined treatment regimen could promote the functional repair of dopaminergic neurons and improve the levels of proteins related to dopamine transport and synthesis. The expression of α -syn in the MPTP group was significantly increased ($P < 0.01$), which was consistent with the pathological characteristics of PD; the expression of this protein in group C was decreased ($P < 0.01$), indicating that the combined treatment could inhibit the abnormal aggregation of α -syn, thereby reducing the pathological damage of PD.

The detection results of apoptosis-related proteins showed that the expressions of Caspase3 and pro-apoptotic protein Bax in the MPTP group were significantly increased ($P < 0.01$), while the expression of anti-apoptotic protein Bcl-2 was significantly decreased ($P < 0.01$). This meant that MPTP induction could activate the cell apoptosis pathway, enhance the pro-apoptotic signal, and reduce the anti-apoptotic ability of cells. After drug administration, compared with the MPTP group, the expressions of Caspase3 and Bax were significantly decreased ($P < 0.01$), and the expression of

Bcl-2 was significantly increased ($P<0.01$). This suggested that the combined treatment could not only inhibit the activation of pro-apoptotic proteins, reduce cell apoptosis, but also up-regulate anti-apoptotic proteins, enhance the anti-apoptotic ability of cells, and thus maintain the survival of neurons.

In the detection of inflammation-related proteins, it was found that the expressions of pro-inflammatory factors such as NF- κ B, TNF- α , and IL-6 in the MPTP group were significantly increased ($P<0.01$), and their expressions were significantly decreased after drug administration ($P<0.01$), indicating that the combined treatment could inhibit the activation of inflammatory pathways and reduce inflammatory damage in the brain. In addition, the expression of NF- κ B inhibitory protein I κ B α in the MPTP group was significantly lower than that in the blank group, and significantly increased after drug administration, which indicated that the combined treatment could inhibit the activation of NF- κ B pathway by up-regulating the expression of I κ B α , thereby exerting anti-inflammatory effects.

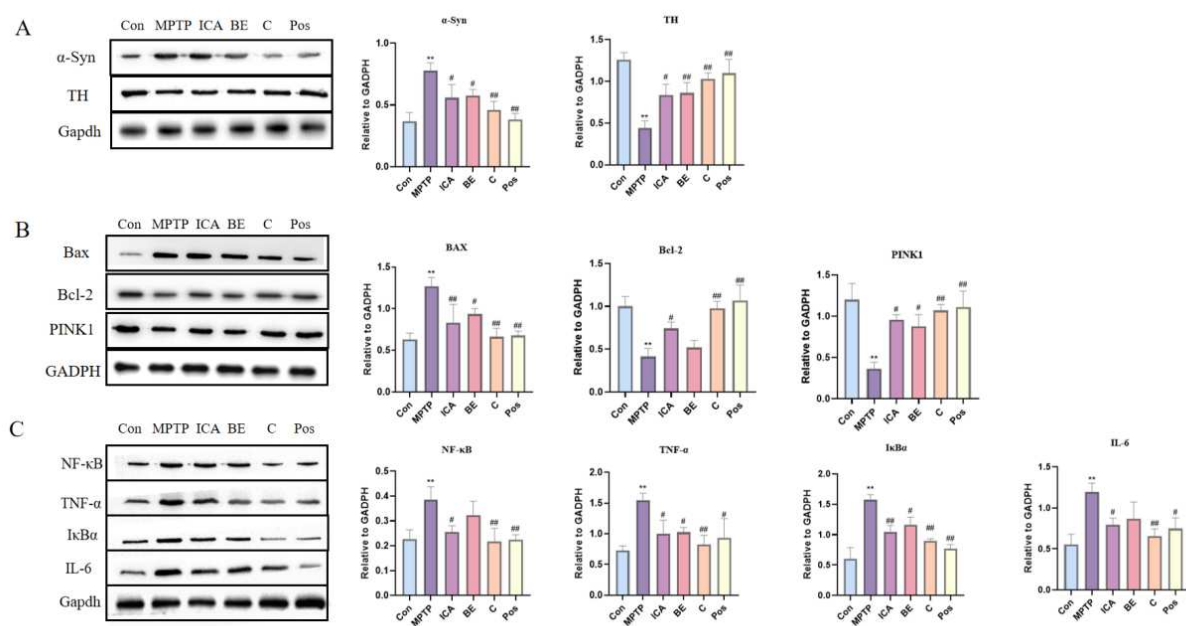


Figure 6 WB results of mouse brain tissue (A) Expression of neuron-related proteins (B) Expression of apoptosis-related proteins (C) Expression of inflammation-related proteins

3.6 Results of 16S rDNA sequencing of mouse intestinal flora

In Figure 8 (A), among all Amplicon Sequence Variants (ASVs), 285 ASVs are unique to the

Con group, 685 ASVs are unique to the MPTP group, and 443 ASVs are unique to the C group. A total of 264 ASVs are shared among the three groups. Figure 8 (B) evaluates the inter-group differences of all samples using the Principal Coordinates Analysis (PCoA) dimensionality reduction method based on the Jaccard distance matrix. From the clustering results of PCoA, it can be observed that the groups represented by different colors are well distinguished; there are significant differences among the six groups ($p < 0.01$). The results of phylum-level analysis are shown in Figure 8 (C, D). The intestinal microbiota of mice is mainly composed of nine taxa. In addition to Firmicutes and Bacteroidetes, they also include Proteobacteria, Actinobacteria, Verrucomicrobia, Patescibacteria, Desulfobacterota, Campylobacterota, and Cyanobacteria. Compared with the Con group, the abundance of Proteobacteria in PD mice is significantly increased, while the abundance of [Note: The original text is missing the name of the phylum whose abundance decreases; please supplement it if necessary] is significantly decreased. However, the combined treatment intervention can reverse the above-mentioned changes in the intestinal microbiota at the phylum level in PD mice. To explore the significantly different species among the groups, we further investigated the effect of combined treatment on the intestinal microbiota of PD mice at the genus level using Linear Discriminant Analysis Effect Size (LEfSe), and the results are shown in Figure 8 (E, F). With $\log \text{LDA} > 3.0$ and $p < 0.05$ as the thresholds for differential analysis, a total of 12 genera with inter-group differences were detected. The genera with the most significant differences in abundance in the Con group are unclassified Bacteroidales, unclassified Peptococcaceae, Rikenella, and Klebsiella. MPTP intervention significantly altered the intestinal microbiota of PD mice, increasing the abundance of Escherichia–Shigella, Enterococcus, and Streptococcus. The genera with the most significant differences in abundance in the C group are Lactobacillus and HT002. The combined treatment regulated the disturbances induced by MPTP in mice to a certain extent. The changes in the abundance of the above-mentioned genera may alleviate PD symptoms in mice by restoring the level of a healthy intestinal microbiota.

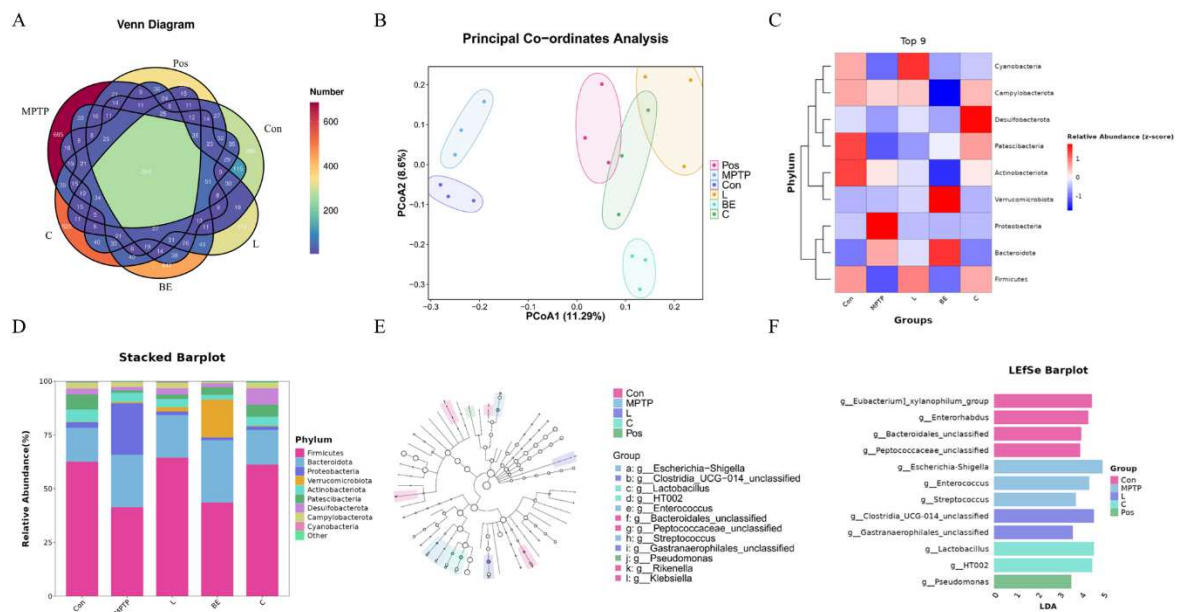


Figure 7 (A) Venn diagram of 16S rDNA sequencing statistics of mouse fecal intestinal flora (B) PCA analysis diagram of mouse fecal intestinal flora (C) Cluster heatmap of relative abundances of nine bacterial phyla (D) Stacked bar chart of intestinal flora at the phylum level (E) LEfSe analysis evolutionary branch diagram at the genus level (F) Distribution bar chart

3.7 Effect on changes in the relative abundance of intestinal microbial communities at the genus level in mice

In MPTP-induced PD model mice, significant abnormal changes occurred in the composition and structure of the gut microbiota. Compared with the control (Con) group, the relative abundances of six bacterial genera in the intestines of mice in the MPTP group—*Ligilactobacillus*, *Akkermansia*, *Lactobacillus*, *Escherichia-Shigella*, HT002, and *Dubosiella*—were decreased, while the relative abundances of *Desulfovibrio* and *Eubacterium* showed an abnormal increasing trend. This state of gut dysbiosis may be closely associated with the pathological progression of PD.

Notably, after combined treatment, the aforementioned trend of gut microbiota disorder was significantly improved: the levels of the six bacterial genera with originally reduced abundances all increased markedly, while the abnormally elevated abundances of *Desulfovibrio* and *Eubacterium* were effectively downregulated, and the overall gut microbiota structure tended to resemble that of the Con group. This result suggests that combined treatment may specifically regulate the abundances of these key bacterial genera in the gut microbiota, reshape the imbalanced gut microecological environment in PD mice, and further exert neuroprotective effects through the bidirectional regulatory mechanism of the "gut-brain axis." This provides potential microbial targets

and intervention strategies for alleviating the pathological damage of PD.

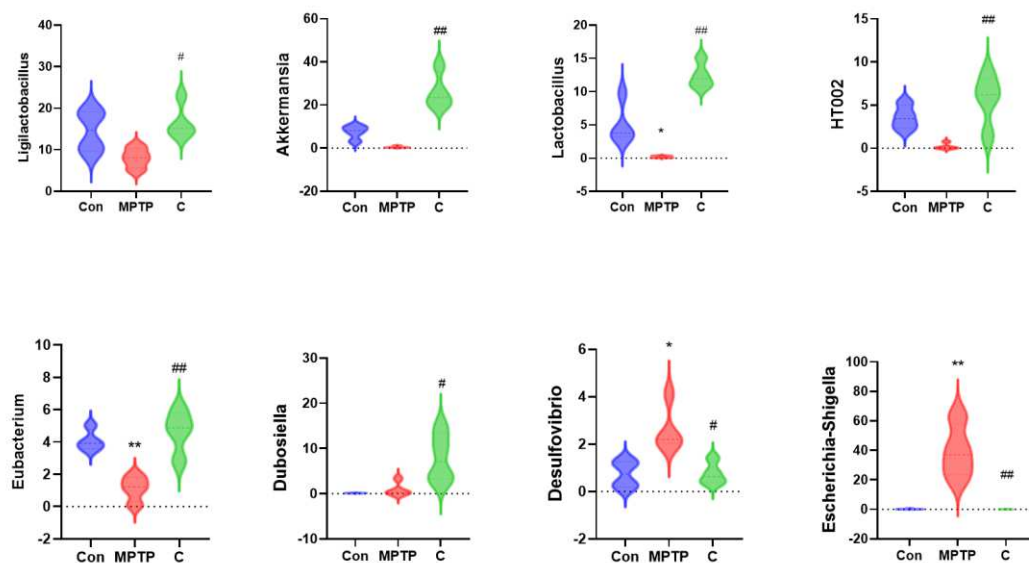


Figure 8 Relative abundance of the gut microbial community at 8 genus level.

3.8 Results of Transcriptomic Analysis of the Substantia Nigra in Mouse Brain Tissue

To evaluate the correlation between the assays, both Spearman and Principal Component Analysis (PCA) correlation methods were employed. Of the total reads, more than 93.8% were successfully aligned to the reference genome, indicating that the sequencing data was sufficient for further analysis. No significant differences were observed between the phases within the treatment groups; however, significant differences were identified between the groups (refer to Figures A and B). Differential gene expression analysis among the groups (Figure C) identified 533 significantly dysregulated genes between Group C and the MPTP group, consisting of 187 upregulated and 346 downregulated genes. Subsequent intersection analysis of differentially expressed genes (DEGs) across the groups identified 114 reversed genes (Figure E), which are hypothesized to represent potential key genes associated with the pathogenesis of PD.

GO and KEGG pathway enrichment analysis was performed on differential genes. The results of GO enrichment analysis showed that the relevant genes may be involved in the functional maintenance and damage repair of dopaminergic neurons in PD by regulating neural signal transmission, immune surveillance, and substance transport across the blood-brain barrier. Among them, the highly significant enrichment of MHC class I presentation of exogenous peptide

antigens (GO:0019886) and microglial activation (GO:0002282) can synergistically aggravate neuroinflammation and neuronal damage, suggesting that these genes are potential targets for immune regulation and neuroprotection in PD.

KEGG pathway analysis indicated that the differentially expressed genes were significantly enriched in pathways related to environmental information processing, human diseases, metabolism, and biological systems. Notably, the majority of these pathways were associated with inflammatory responses, oxidative stress, and α -syn pathology. The Ras signaling pathway has been identified as a critical component in the pathogenesis of PD, with its dysregulated activation or inhibition being intricately associated with disease mechanisms. In PD, oxidative stress and mitochondrial dysfunction can activate the Ras pathway, while excessive ROS may directly modify Ras proteins, resulting in sustained activation and subsequent apoptosis via the ERK signaling cascade.

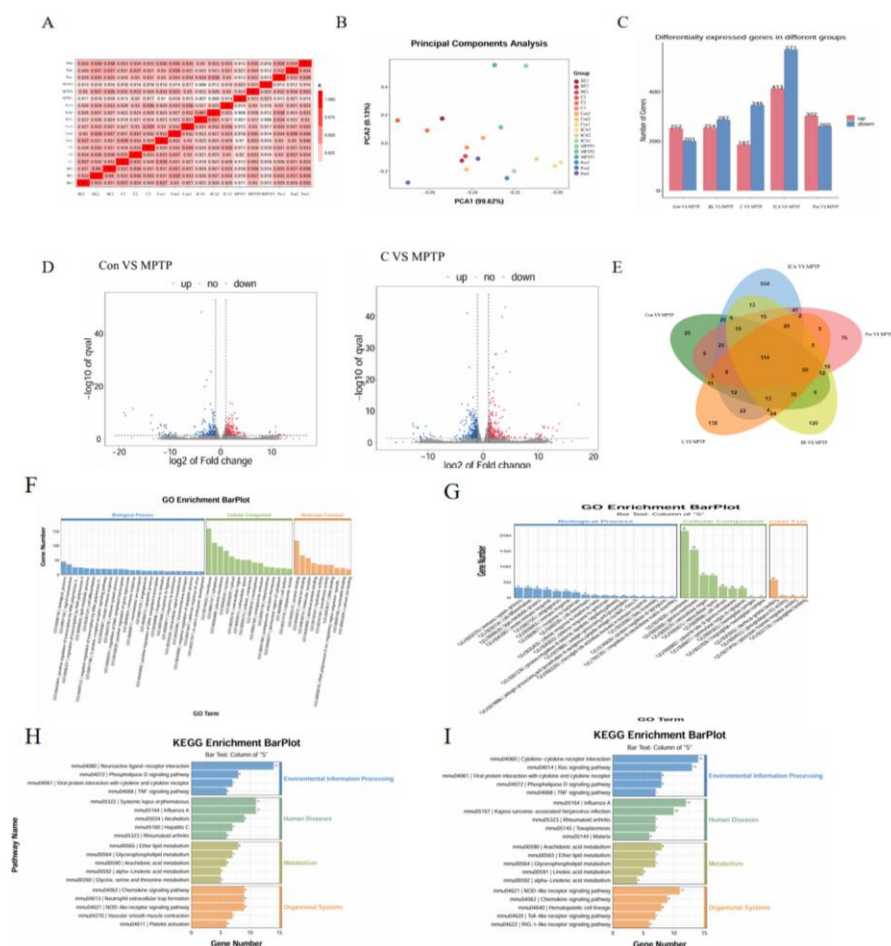


Figure 9 Correlation analysis and differential gene expression (A) Spearman correlation analysis (B) PCA correlation analysis (C) Expression of differential genes between different groups and the model group (D) Volcano plot of differential gene expression between different groups and the model group (E) Venn diagram of differential genes between the model group and each group (F)

GO: MPTP group vs. Con group (G) GO: MPTP group vs. C group (H) KEGG: MPTP group vs. Con group (I) KEGG: MPTP group vs. C group

3.9 RT-qPCR

This study demonstrates that the combination therapy of icariin and probiotics alleviated motor dysfunction in MPTP-induced PD mice by modulating the Ras signaling pathway, as indicated by the upregulation of key genes, including *Epha2*, *Angpt2*, *Tgfa*, and *Pla2g4a*. These findings propose that targeted regulation of the Ras pathway could serve as a promising therapeutic strategy for PD intervention. Validation through RT-PCR analysis (Figures A-D) revealed significantly elevated mRNA levels of *Epha2*, *Angpt2*, *Tgfa*, and *Pla2g4a* in the MPTP group compared to the control group ($p < 0.01$). Although ICA and BE treatments did not significantly downregulate *Epha2* and *Angpt2* expression, both the C and Pos groups exhibited marked suppression of these genes ($p < 0.01$). The differential regulation observed suggests a potential role in disease progression through the modulation of neuroinflammatory responses and neuronal survival states, which may, in turn, affect the integrity of the blood-brain barrier. Notably, the expression of *Tgfa* and *Pla2g4a* was significantly reduced by interventions with ICA, C, and Pos ($p < 0.01$). This downregulation is likely to contribute to the neuroprotection of dopaminergic neurons by either mitigating their degeneration or suppressing the production and release of inflammatory mediators that exacerbate neuroinflammation and neuronal injury. The distinct regulatory patterns observed across treatment groups suggest that different therapeutic strategies may exert disease-modifying effects in Parkinson's disease through the selective modulation of these pathogenic processes.

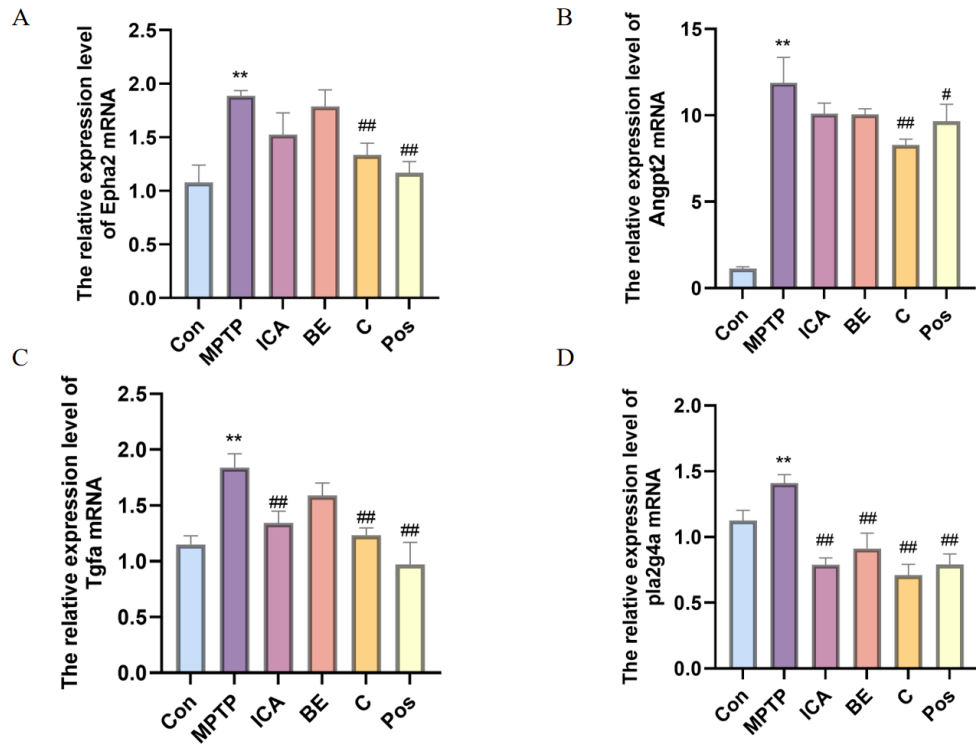


Figure 10 Gene Expression Level (A) Expression level of Epha2 gene; (B) Expression level of Angpt2 gene; (C) Expression level of Tgfa gene; (D) Expression level of pla2g4a gene.

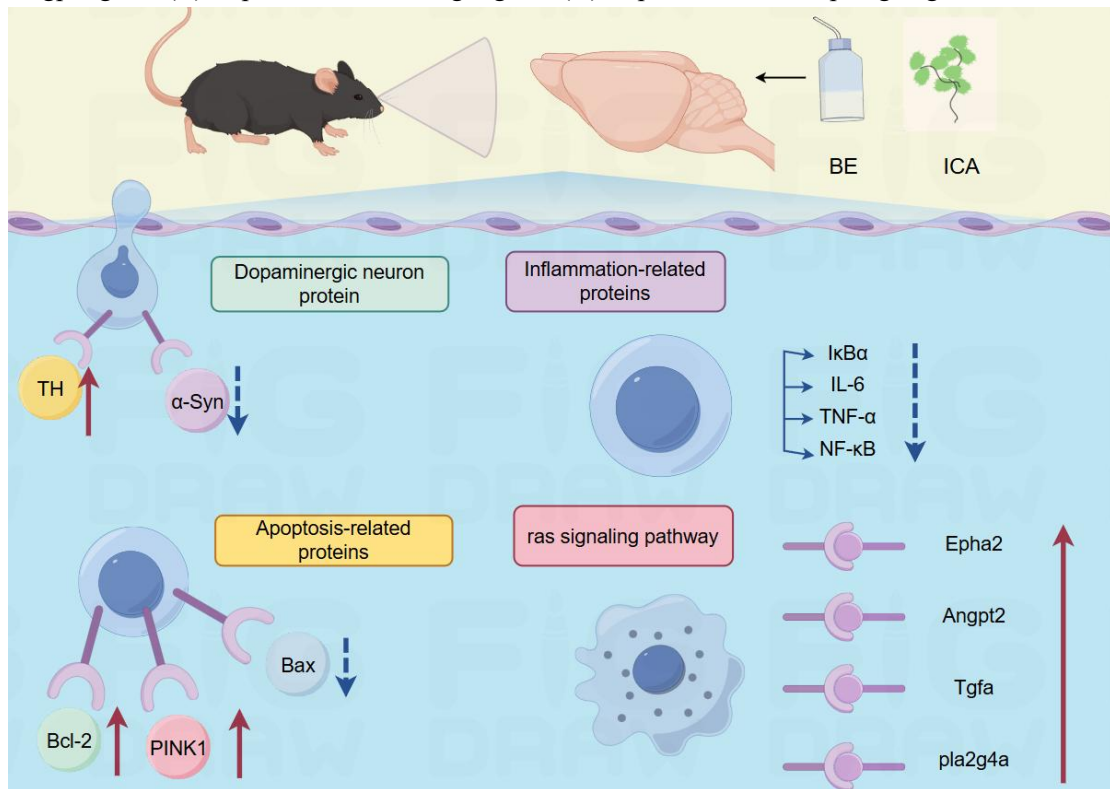


Figure 11 Molecules significantly increased after combined treatment with Icarin and probiotics are marked with red arrows, while those significantly decreased are marked with blue font.

4 Discussion

As a common neurodegenerative disease, PD has a pathogenesis closely associated with gut microbiota dysbiosis and gut-brain axis imbalance. Specifically, abnormal gut microbiota composition can trigger intestinal-derived inflammation, disrupt intestinal barrier integrity, and induce central nervous system (CNS) inflammation via the gut-brain axis, ultimately leading to dopaminergic neuron damage[18]. ICA, a natural bioactive component, has been proven to alleviate neuroinflammation and improve cognitive function in PD models[19]. However, the effect of ICA combined with probiotics on PD related to gut microbiota dysbiosis has not been reported. In this study, ICA was administered in combination with *Enterococcus faecium* and *Bacillus subtilis* to intervene in MPTP-induced PD mice. The results showed that this combined strategy could improve motor function and dopaminergic neuron damage in mice by reshaping gut microbiota, regulating gut-brain axis signaling, and reducing oxidative stress and neuroinflammation, thereby providing a new perspective for multi-targeted intervention in PD.

Motor dysfunction is a key characteristic of PD. The MPTP-induced PD model is a commonly used animal model that well mimics the motor symptoms of PD patients. In this study, ICA combined with probiotics was demonstrated to ameliorate MPTP-induced motor deficits, as confirmed by the pole test, swimming test, and open field test. Motor deficits are usually associated with histopathological changes: the loss of dopaminergic neurons is linked to motor impairments, leading to progressive degeneration in PD.

Commonly used indicators reflecting oxidative stress include Malondialdehyde (MDA), (SOD, and CAT. Among them, MDA can reflect the degree of lipid peroxidation, oxygen free radical metabolism, and oxidative damage in the body; SOD exerts antioxidant effects by scavenging superoxide anion radicals; and CAT prevents cellular damage by eliminating oxygen free radicals[20]. Inflammatory cells can release large amounts of reactive oxygen species (ROS), which further exacerbate oxidative stress. Excessive ROS production or decreased antioxidant capacity in the body leads to oxidative stress, which in turn promotes lipid peroxidation and increases MDA production [21]. GSH is an important antioxidant in the brain, and increasing GSH levels in the brain is one of the effective therapeutic strategies for PD[22]. The loss of DA-producing neurons in the substantia nigra pars compacta (SNpc) is a defining pathological hallmark of PD. Single-nucleus RNA sequencing (snRNA-seq) analysis of SNpc samples from elderly controls and patients with

PD or Lewy body dementia (LBD) revealed a significant reduction in the proportion of DA neurons[23]. Consistent with this finding, the combined treatment in our study enhanced the antioxidant capacity of the CNS, thereby helping PD mice alleviate oxidative stress and increase DA levels.

Inflammatory responses play a crucial role in the development and progression of PD. Numerous studies have shown that these inflammatory factors are also highly expressed in the brain tissues of PD patients[24]. The massive release of inflammatory factors can activate microglia and astrocytes, thereby triggering neuroinflammatory responses and causing damage to dopaminergic neurons [25]. Abnormal aggregation of α -Syn is one of the main pathological features of PD; it can promote the release of inflammatory factors, forming a vicious cycle that further aggravates neuroinflammation and neuronal damage[26]. A study indicated that overexpression of α -Syn in PD mice exacerbated parkinsonian symptoms, and α -Syn aggregation was detected in both the intestine and brain[27]. Therefore, it is hypothesized that intestinal damage may also play an important role in the progression of PD symptoms. In PD patients, intestinal α -Syn accumulation was found to increase intestinal permeability, leading to elevated levels of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) in the blood and brain. High TLR4 expression is associated with microbial dysbiosis, intestinal inflammation, intestinal barrier disruption, bacterial translocation, microglial activation, and neurodegeneration — suggesting that intestinal barrier integrity is closely related to neuroinflammation, and increased intestinal permeability may raise the risk of PD.

In dopaminergic neurons, TH is a rate-limiting enzyme for DA synthesis; a decrease in TH content or abnormal TH function directly impairs DA secretion. Perez et al.[28] found that overexpression of α -Syn inhibited TH dephosphorylation, reduced TH activity, and decreased DA levels. In this study, Western blot analysis showed that the α -Syn content in the brain tissues of mice in the MPTP group was significantly increased, while the TH content was significantly decreased. In contrast, compared with the MPTP group, mice receiving the combined treatment exhibited decreased α -Syn content and significantly increased TH content in brain tissues, implying that the combined treatment exerts a certain neuroprotective effect. A recent study reported that α -Syn in peripheral blood can activate immune cells in the blood [29], indicating that α -Syn may drive the immune system toward a pro-inflammatory response, and a series of inflammatory

reactions may in turn affect PD. Excessive inflammatory responses lead to the massive release of inflammatory factors, which further disrupt the blood-brain barrier (BBB) and impair mitochondrial function. Inflammatory factors can also serve as potential biomarkers and targets for regulating immune responses in PD. Researchers found that levels of TNF- α , IL-6, and IL-1 β were significantly higher in PD patients compared with healthy individuals[30].

The NF- κ B signaling pathway is central to the regulation of inflammatory responses. When the NF- κ B signaling pathway is activated, it promotes the transcription and expression of inflammatory factors, thereby exacerbating inflammatory reactions[31]. As a key enzyme for DA synthesis, the expression level of TH directly affects DA production. The significant downregulation of TH expression in the brains of MPTP-induced mice is fully consistent with the pathological characteristics of PD patients, which include degeneration and loss of dopaminergic neurons and reduced DA synthesis[32]. The damage and loss of dopaminergic neurons cause typical PD symptoms such as bradykinesia and tremors. The results of this study further confirm the important roles of inflammatory responses, the NF- κ B signaling pathway, mitochondrial function, and cell apoptosis in the pathogenesis of PD.

Gut microbiota not only participates in processes such as human metabolism and maintenance of normal immune function but also has a close association with neurological disorders such as anxiety and depression. Existing studies have shown that abnormal increases in Proteobacteria are associated with adverse physiological states, including impaired intestinal barrier function and exacerbated inflammatory responses[33]. Conversely, decreased abundance of phyla such as Actinobacteria may disrupt normal metabolic processes and immunomodulatory functions in the gut. Some studies have noted that certain genera within Actinobacteria are involved in metabolic activities such as vitamin synthesis in the gut; a reduction in their abundance may decrease the production of these beneficial metabolites [34]. The combined treatment intervention was able to reverse these phylum-level changes in the gut microbiota of PD mice, suggesting that this therapeutic approach plays a positive role in restoring gut microbiota balance.

Genus-level analysis using Linear Discriminant Analysis Effect Size (LEfSe) revealed that an increase in *Escherichia-Shigella* may lead to elevated toxin production in the gut, trigger inflammatory responses, and thereby affect nervous system function[35][36]. *Lactobacillus*, a representative genus of probiotics, exerts multiple beneficial effects such as regulating intestinal

immunity and improving intestinal barrier function [37][38]an increase in its abundance may help restore gut microecological balance and reduce inflammatory responses, thereby exerting neuroprotective effects in PD mice. Akkermansia has been reported to be closely associated with functions such as maintaining intestinal barrier integrity and regulating metabolism[39][40]Some species within the genus Eubacterium are involved in the production of short-chain fatty acids (SCFAs) in the gut; SCFAs are of great significance for maintaining intestinal homeostasis and regulating immunity, and a decrease in their abundance may disrupt intestinal homeostasis[41]. Changes in the abundance of these genera may indirectly alleviate PD symptoms in mice by restoring healthy gut microbiota levels and regulating intestinal immune and metabolic functions. However, the specific mechanism of interaction between gut microbiota and PD requires further in-depth investigation.

Epha2, a member of the Eph receptor family, may have increased expression associated with the activation of inflammatory signals following dopaminergic neuron damage. Previous studies have shown that abnormal activation of the Ephrin-Eph signaling pathway can promote microglial activation and neuroinflammatory responses[42]. The upregulation of Angpt2 may reflect BBB dysfunction — in PD, Angpt2 disrupts tight junctions between vascular endothelial cells, exacerbating inflammatory cell infiltration [43].Regarding the upregulation of Transforming Tgfa and Pla2g4a, their mechanisms may be related to oxidative stress and inflammatory cascades in neurodegeneration. As a member of the epidermal growth factor family, Tgfa is considered to have neuroprotective effects in some studies; however, its abnormal elevation in PD models may indicate excessive activation of glial cells[44]. Pla2g4a encodes cytosolic phospholipase A2 (cPLA2 α), a key enzyme in the synthesis of inflammatory mediators. Increased expression of Pla2g4a can promote arachidonic acid metabolism, exacerbate oxidative stress and neuronal apoptosis, which is consistent with the mechanism of MPTP-induced dopaminergic neuron damage[45].

In summary, the combined intervention of Icariin and probiotics exerts a significant anti-PD effect through multi-link regulation of the “gut microbiota – gut-brain axis – central inflammation/oxidative stress” pathway. This synergistic model of “traditional Chinese medicine (TCM) monomer + probiotics” not only leverages the anti-inflammatory and antioxidant advantages of Icariin but also utilizes the gut microecological regulatory effects of probiotics. It provides a new strategy with both safety and targeting for the preventive intervention and treatment

of PD, and its clinical translation potential deserves further exploration.

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