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# Wumei-Pill Alleviates Parkinson's Disease Induced by Dopamine Neuron Damage in Mice by Regulating the Gut-Brain-Microbiome Axis

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## ABSTRACT

**Objective:** Investigate the protective effects and the ways WuMei-Pill (WMP) work on Parkinson's disease (PD), focusing on its ability to restore the PD-induced gut microbiota imbalance and elucidate the underlying mechanisms.

**Methods:** This study employed LPS-induced inflammation BV2 cells and a PD mouse model induced by 6-OHDA to explore the treatment of WMP. A series of assessments were performed, encompassing behavioral evaluations, immunohistochemistry, Western blot (WB), as well as enzyme-linked immunosorbent assay (ELISA), to evaluate neurological function of WMP on PD. Fecal samples as well as brain tissues were collected for microbiome and transcriptome analysis.

**Results:** WMP treatment elevated neurological function, increased the number of TH+ cells, as well as brain dopamine levels. 6-OHDA-induced dopaminergic neuronal damage was connected to an increase in pro-inflammatory bacteria (Bacteroides), bacteria involved in tryptophan metabolism (Azospirillum_sp.47_25 as well as unclassified Bacteroidia), as well as cholesterol metabolism (unclassified [Eubacterium]_coprostanoligenes_group), while the number of anti-inflammatory bacteria (Roseburia) was reduced. The WMP study investigated the shifts in the microbiota and changes in key brain metabolites, including L-tryptophan and Bambuterol.

**Conclusion:** Dopaminergic neuron damage-induced microbiome dysbiosis worsen Parkinson's disease symptoms. WMP corrected these microbial and metabolic imbalances, reducing dopaminergic neuron loss, increasing dopamine levels, and improving neurological function.

## INTRODUCTION

After Alzheimer's disease, Parkinson's disease (PD) is the second most prevalent neurological illness. Slowness of movement like tremors, muscle rigidity, as well as postural instability are characterized as the motor symptoms of PD Bloem et al. (2021). By 2016, more than six million people globally were living with Parkinson's, with estimates suggesting that the number could double in the next generation Dorsey et al. (2018). The core pathophysiology involves the death of dopaminergic neurons in the substantia nigra as well as the formation of α-synuclein aggregates in those neurons. The hallmark motor symptoms of PD include rigidity, bradykinesia, resting tremors, as well as muscle spasms. Additionally, non-motor symptoms like sleep disturbances, depression, as well as cognitive impairments are also prevalent Di Stefano et al. (2021)., Aarsland et al. (2021). Currently, dopamine replacement therapy, particularly using Levodopa, remains the most effective treatment option Fox et al. (2018).

However, these medications merely alleviate symptoms without halting or reversing the disease, underlining the urgent need for alternative therapeutic strategies. Recent advancements in grasping the underlying mechanisms of PD have caused the identification of new therapeutic targets. For instance, P2B001, a new drug that combines Pramipexole sub-therapeutic doses (a dopamine agonist) and Rasagiline (an MAO-B inhibitor), has shown promising results in phase II clinical trials by reducing the adverse effects typically associated with dopaminergic treatments Fox et al. (2018). Furthermore, Tavapadon (PF-06649751), a selective D1/D5 dopamine receptor agonist, has demonstrated the potential to mitigate side effects such as blood pressure fluctuations and impulse control disorders (ICDs). Riesenberg et al. (2020). These emerging clinical trials offer exciting possibilities for discovering new PD treatments that could provide distinctive therapeutic

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benefits. PD's progression is marked by the degeneration of dopaminergic neurons in the substantia nigra, a critical brain area involved in motor control. Pro-inflammatory cytokines, like TNF-alpha, IL-1beta, as well as IL-6, are observed at elevated levels in the brains of PD patients, playing a critical part in the neuroinflammatory processes associated with the disease Tansey et al. (2022). In conclusion, inflammation plays a central and multifaceted role in PD's pathogenesis, making it an important focus for the development of more effective and targeted therapies.

Traditional Chinese medicine (TCM) enjoys a long history of treating various ailments, with proven efficacy, safety, and affordability, especially through its holistic approach to chronic diseases Wang et al. (2018). By utilizing natural remedies refined over centuries, TCM offers valuable therapeutic strategies for Parkinson's disease, enhancing patient outcomes by addressing both symptom management and the modulation of disease progression Wang et al. (2021). This dual treatment approach emphasizes the integral role that TCM can play within the broader context of PD treatment options, aligning time-honored methods with modern medical needs. Wumen Pill, a formulation containing several herbs traditionally used to treat conditions like internal heat, chronic cough, and parasitic infections, has been found to influence immune functions in relevant animal models Duan et al. (2023), Lu et al. (2024). Studies show that Wumen Pill (WMW) lowers the immune infiltration of myeloid-derived suppressor cells in early stages and enhances CD4+ as well as CD8+ T cell populations in the spleens of CAC mice, thereby reshaping the immune microenvironment Lu et al. (2024). However, the interaction between Wumen Pill and conventional PD medications, particularly in the context of neuroinflammation, is not yet well understood.

This study uses a PD mouse model, induced by dopaminergic neuron damage, to explore the preventative potential of Wumen Pill against PD. Employing a combination of behavioral assessments, molecular biology, and multi-omics approaches, the research will examine how Wumen Pill may influence gut-brain axis dysbiosis, a key feature in PD. By doing so, the research aims to find new therapeutic targets as well as strategies for preventing as well as treating PD, highlighting the promising role of Wumen Pill in advancing these efforts.

## MATERIAL AND METHODS

### Cells

Mouse microglial BV2 cells were sourced from the Shanghai Cell Bank (China) as well as cultured in DMEM (Wuhan PNLS Life Science Technology Co., Ltd.), supplemented with 10% fetal bovine serum (FBS; Suzhou EcoScience Biotechnology Co., Ltd.) as well as 1% antibiotics (penicillin 100 **U**/ml, streptomycin 100 U/ml; Adamas Life). The cells were maintained in a humidified incubator at 37°C with 5% CO2. Experiments were performed once the cells entered the logarithmic growth phase.

### Cell Viability Assay

BV2 cells in the logarithmic growth phase were seeded in 96-well plates ($6\times10^3$ cells/well) as well as incubated at 37°C for 12 h to allow full adherence. Various concentrations of LPS (0, 5, 10, 20, as well as 40 μg/ml) were incorporated for 24 h, and cell viability was assessed via the CCK-8 assay (Adamas Life). The resulting data were used to generate an IC50 curve, yielding an IC50 value of 9.5 μg/ml. For subsequent experiments, BV2 cells were treated with 9.5 μg/ml LPS for 24 h, followed by exposure to different concentrations of WuMei Pill (WMP) for another 24 h. After the incubation period, CCK-8 reagent was incorporated, as well as the plates were incubated at 37°C for 2-4 hours. Absorbance at 460 nm was then measured to assess cell viability.

### Animals

Fifty healthy male C57BL/6 mice, aged 10 weeks as well as weighing between 25-30 grams, were acquired from the Animal Experiment Center at Kunming Medical University. The mice underwent a one-week acclimatization period under standard laboratory conditions before being divided into 4 experimental groups: Sham, Sham+WMP, PD model, as well as PD+WMP, with 10 mice per group. Following the modeling procedure, WMP was administered intragastrically as outlined in Figure 1A. The Animal Experiment Ethics Committee of Kunming Medical University provided ethical permission for this work (approval code: KMMU20231408), and all procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

### Model Creation and WuMei Pill (WMP) Administration

**6-OHDA Unilateral Lesion Procedure:** Mice allocated to the PD model group underwent a 24-hour fasting period, with free access to water. After anesthesia, a craniotomy was performed, and a 2 μl volume of 6-OHDA (5 mg/ml; Aladdin, China) was injected into the left striatum using a stereotaxic apparatus (coordinates: AP: +0.3; ML: +2.3; DV: -2.9) at a rate of 0.5 μl/min. post-surgery, the mice were kept warm for 2 hours before being returned to their cages for recovery monitoring.

**WMP Treatment Protocol:** WMP was administered intragastrically at a dosage of 0.1 mg/g body weight in a 200 μl volume daily, starting one week before the modeling procedure as well as continuing throughout the study, as shown in Figure 2A and B. Beginning in the second week after surgery, the mice received an intraperitoneal injection of apomorphine

solution (0.5 μg/g body weight; A276058, Aladdin, China). After administration, the mice were placed in a clean, sterile chamber, and their rotational behavior was observed for 30 minutes. The number of rotations towards the non-lesioned side was recorded, with more than 7 rotations per minute considered indicative of successful lesioning. This test was carried out weekly until one week before sample gathering, as outlined in Figure 2B.

**Preparation of Brain Tissue Samples**

After perfusion fixing, brain tissues were extracted from 10 mice in each experimental group. In addition, the left and right hemispheres of the brains of 5 more mice per group were separated. To maintain the integrity of the proteins, these hemispheres were lysed via RIPA lysis buffer that had been enhanced with 1% PMSF. After lysis, tissue samples were stored at -80°C for subsequent analyses.

**Conduct of Behavioral Assessments**

**Pole Climbing Test:** Each mouse was placed atop a 55 cm pole, and the time taken to descend was recorded. This procedure was repeated three times for each animal. The mean of these trials was computed and used as the experimental data.

**Rota-Rod Test:** Mice were given 30 minutes to acclimatize to the test environment. Over the course of three consecutive days, each mouse underwent training sessions with the rotating rod at speeds of 4 rpm, 8 rpm, and a gradual acceleration from 4 rpm to 16 rpm, with each session lasting 5 m. The maximum time each mouse was able to remain on the rod was recorded for later analysis.

**Cylinder Test:** After a 30-minute acclimatization period, each mouse's interaction with the cylinder walls, including the number of wall touches in 10 minutes, was recorded. The test was conducted three times per mouse. The average value was used for analysis.

**Immunohistochemical Analysis**

Mouse brain samples were fixed in formaldehyde, dehydrated via an automated process, embedded in paraffin, as well as sectioned. Deparaffinization, rehydration, and heat- mediated antigen retrieval in a sodium citrate solution were performed on the sections (Catalog No. D0081, Beyotime, China). Peroxidase Blocking Solution (Catalog No. P0100A, Beyotime, China) was used to block endogenous peroxidase activity, and 10% goat serum was used to reduce non-specific binding for an hour at room temperature. After that, the sections were incubated at 4 °C for the whole night with a primary antibody that targets TH. The tissue slices were cleaned the next day and treated for 30 minutes at 37°C with a secondary antibody. After counterstaining with hematoxylin, DAB (3,3'-Diaminobenzidine) staining was performed via a DAB Staining Kit and seen using a Leica microscope.

**Western Blotting**

Protein samples were denatured by heating them to 100°C for 10 m while also being combined with 5x loading buffer. After being separated using SDS-PAGE, the samples were transferred to a PVDF membrane. Main antibodies against TH as well as β-actin were used to incubate the membrane, and then a secondary antibody was added. The membrane's purpose was to detect signals.

**ELISA Assay**

BV2 cells were seeded in 6-well plates ($1\times10^6$ cells/well) as well as cultured for 12 hours. The cells were then exposed to LPS (9.5 μg/ml) for 24 hours and treated with varying concentrations of WMP for an additional 24 hours. The concentrations of interleukin (IL)-6 (Catalog No. ml098430), IL-10 (Catalog No. mIC50274-1), and TNF-α (Catalog No. ml002095) were quantified using ELISA kits (Shanghai Enzyme Linked Biotechnology Co., Ltd.). Absorbance was measured at 450 nm following the manufacturer's instructions.

**Microbiome and Metabolome Analysis**

At the 7th week after the modeling procedure, fresh fecal samples were gathered (including the Sham group, PD model group, as well as PD+WMP group) for microbiome analysis. Brain tissue samples from the groups of mice were also collected for metabolomics analysis. Both microbiome and metabolomics were accomplished by Beijing Biomarker Technology Company in China for comprehensive microbiome and metabolomics analysis.

Trimmomatic v0.33 was a classical software to refine the raw sequence data in the initial process of microbiome analysis, and cutadapt software 1.9.1 employed to indentify primer sequences by generation of clean sequences devoid of any primer sequences. DADA2 (Divisive Amplicon Denoising Algorithm 2), an algorithm used for processing high-throughput 16S rRNA gene or other amplicon data, was applied to analyze microbiome diversity. It recover higher-resolution microbial community data by removing noise, providing noise-free, true sequences by eliminating sequencing errors Bolyen et al. (2019), Callahan et al. (2016), following steps as: Facilitated the denoising of the data, Producing a final gathering of sequences that were free from chimeric artifacts,The processed data was then subjected to various analyses,Classification of microbial features (e.g., Operational Taxonomic Units [OTUs], Amplicon Sequence Variants, Diversity assessments (alpha diversity / beta diversity / Shannon index),Differential abundance testing (DESeq2 ), Correlation research, Predictive

functional analyses (PICRUSt or FAPROTAX).

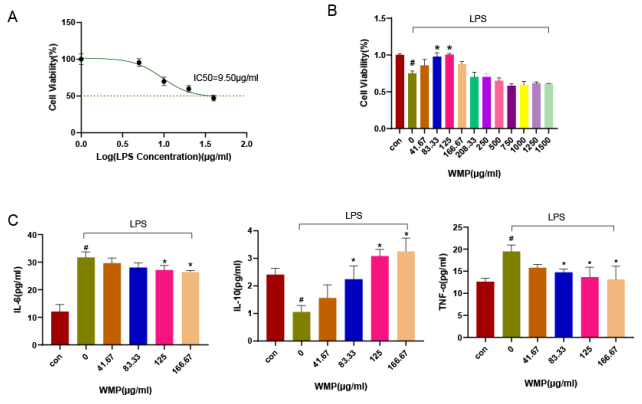
## RESULTS

### WMP effectively reverses LPS-induced inflammation in BV2 cells

Microglia, as the intrinsic immune cells of the brain, have a crucial role in maintaining the homeostasis of the brain microenvironment and are often utilized to establish cellular models of PD). Accordingly, we first determined the IC50 of LPS on BV2 cells: 9.5 μg/mL Figure 1A. Subsequently, BV2 cells were stimulated with LPS (9.5 μg/mL) for 24 h to simulate the PD disease model, based on which we evaluated the therapeutic effects of WMP on PD. Following treatment with different concentrations of WMP for 24 hours, we found that WMP at concentrations of 83.33 μg/mL as well as 125 μg/mL significantly reversed the decline in BV2 cell viability induced by LPS compared to the control group Figure 1B.

Excessive production of pro-inflammatory cytokines damage central nervous system tissues as well. Therefore, we employed ELISA to measure the levels of pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokine IL-10 (Fig 1C). BV2 cells were stimulated with LPS (9.5 μg/mL) for 24 hours, followed by treatment with WMP (0, 41.67, 83.33, 125, 166.67 g/mL) for 1 hour. The levels of IL-6 as well as TNF-α were remarkably elevated in the LPS-stimulated BV2 cell group compared to the control one. Yet, WMP reduced the levels of pro-inflammatory cytokines IL-6 as well as TNF-α in a concentration-dependent manner while increasing the level of IL-10.

**Figure 1:** Wumen Pill attenuates LPS-induced inflammatory response in BV2

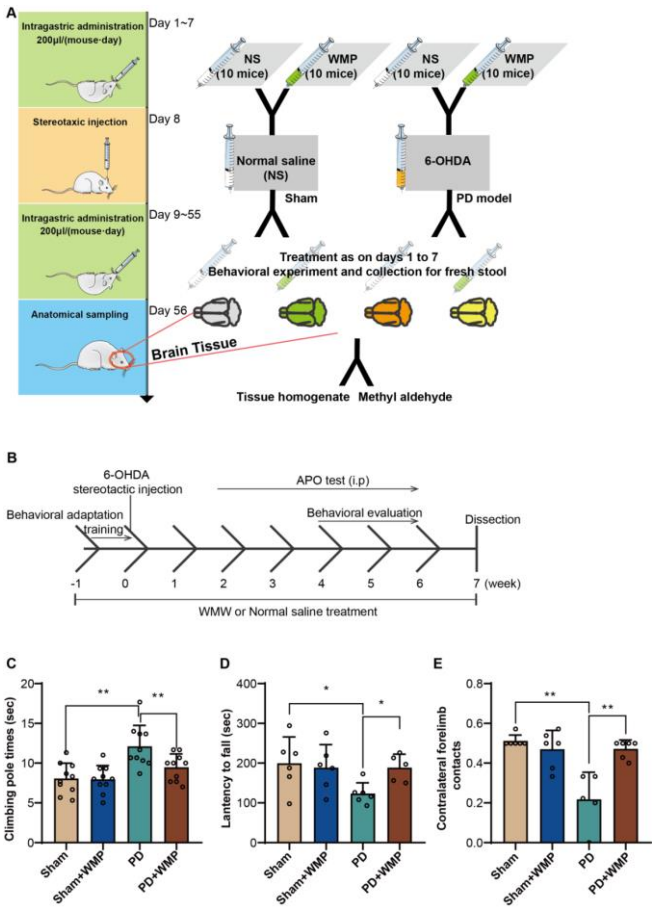



(A) Half maximal inhibitory concentration of BV2 by LPS (CCK-8). (B)WMP enhances the vitality of BV2 cells induced by LPS (CCK-8). (C) WMP decreases the levels of IL-6 and TNF-α in BV2 cells stimulated by LPS, and increases the level of IL-10. Data are presented as mean±SD of three independent experiments. #P<0.01 vs. control group; *P<0.01 vs saline treatment alone. LPS treatment group. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor.

### Improvement of PD Symptoms through Wumei pill (WMP) Treatment

The mice in the PD group showed a remarkable drop in the number of wall touches with the contralateral forelimb (P<0.01 compared to Sham, shown in Figure 2C, a remarkable decrease in the duration of stay on the rotarod (P<0.05 compared to Sham, shown in Figure 1B, as well as a marked increase in the time needed to descend the climbing pole (P<0.01 compared to Sham, as shown in Figure 2A. PD+WMP mice, on the other hand, showed a significant decrease in climbing pole descent time (P<0.01 compared to the PD one, as shown in Figure 2A, a significant rise in rotarod duration (P<0.05 compared to the PD one, as shown in Figure 2B, as well as a greater frequency of wall touches with the contralateral forelimb (P<0.01 compared to the PD one, as shown in Figure 2C following WMP treatment.

**Figure 2:** Overview of the Animal Model




(A) Classification of animals and treatment protocols. (B) Timeline of experimental procedures. (C) Assessment using the pole climb test. (D) Evaluation with the rotating rod test. (E) Analysis through the cylinder test. Significance levels are denoted as *p<0.05, **p<0.01.

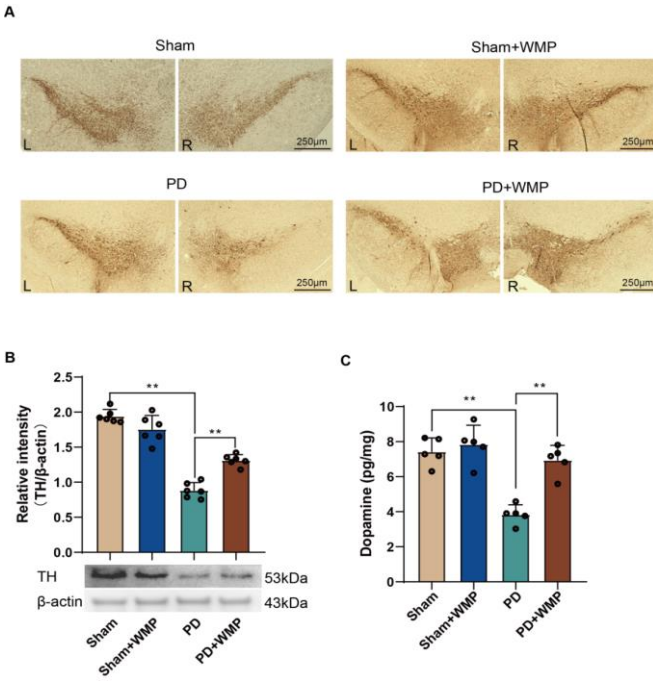
### Wumei Pill prevents PD by protecting substantia nigra as well as its dopamine synthesis function

Differential expressions across the research groups were

shown by immunohistochemistry using TH antibody staining Figure 3A. Strong TH-positive staining was seen in the Sham as well as Sham+ WMP groups, suggesting that dopamine production was functioning normally. Mice from the PD model group displayed a significant reduction in TH expression in the right substantia nigra. Interestingly, the PD+WMP therapy one showed consistent TH expression in both substantia nigra hemispheres, suggesting that the tablet had protective effects on dopaminergic neurons.

Additionally, PD-afflicted mice's brains showed significantly lower levels of dopamine and TH protein ($P<0.01$ compared to Sham, Figure 3B as well as C). WMP treatment, yet, resulted in a remarkable rise in dopamine and TH protein levels ($P<0.01$ compared to PD, Fig 3B as well as C), highlighting the pill's capacity to lessen PD-related biochemical alterations.

**Figure 3:** WMP Mitigates the Loss of Dopaminergic Neurons Induced by 6-OHDA

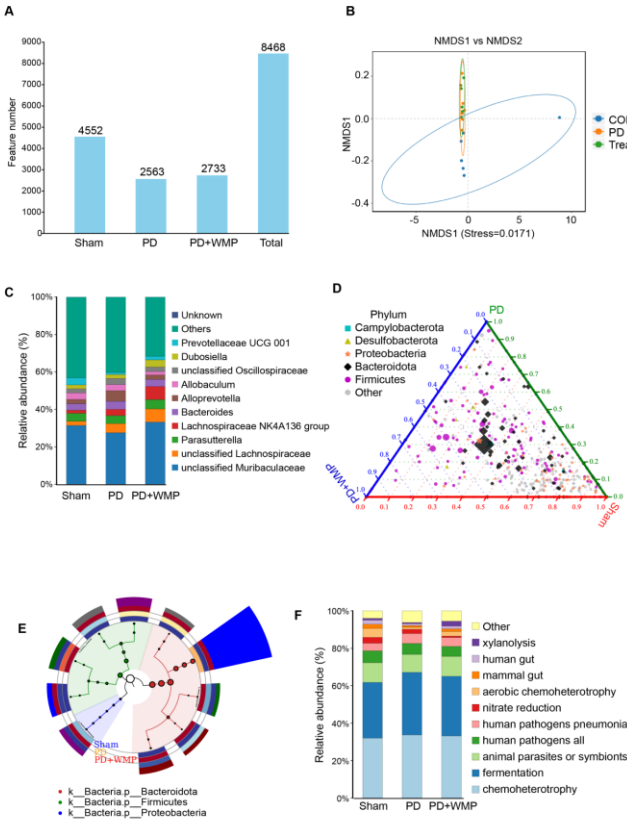



(A) Immunohistochemical analysis of TH protein in the substantia nigra. "L" denotes the left hemisphere, and "R" represents the right hemisphere of the brain. (B) Quantification of TH protein levels in the right hemisphere. (C) Measurement of dopamine levels in the right hemisphere via ELISA. Significance levels are indicated as $^{**}P<0.01$. The scale bar is set at 250μm.

**Integral analysis of gut flora after treatment by Wumei Pill in PD model**

PD has been linked to changes in the microbial ecosystem, according to recent research. We evaluated the effect of WMP on gut microbiota in our study. There were not many variations of the PD with PD-Wumei treated groups, according to the research. However, the PD as well as PD-Wumei treated ones showed a substantial decrease in total OTUs as compared to the Sham group (Figure 4A). The PD as well as PD-Wumei samples grouped closer together, indicating a higher degree of similarity between them, according to Nonmetric Multidimensional Scaling (NMDS) analysis (Figure 4B). Interestingly, the species distribution study showed the PD as well as PD-Wumei groups differed significantly in terms of microbial abundance. In particular, elevated levels of Alloprevotella were seen in the PD group, suggesting a possible association with Parkinson's disease. Lachnospiraceae_NK4A136_group remarkably increased in the PD-Wumei one, suggesting a possible correlation with the therapeutic advantages of WMP in PD (Figure 4C). Bacteroidota, which was positioned in the middle, shifted toward the Ctrl as well as PD groups, whereas Proteobacteria and Firmicutes leaned toward the Ctrl as well as PD-Wumei groups, respectively, according to the ternary phase diagram analysis (Figure 4D). Furthermore, Bacteroidota, Firmicutes, as well as Proteobacteria were validated as the primary phyla by graph-based phylogenetic analysis (Figure 4E). PD-Wumei therapy particularly boosted xylanolysis-associated microbiota, whereas PD condition improved fermentation-related microbiota, according to the FUNGuild functional prediction (Figure 4F). All together, these results imply PD alters the typical makeup of gut flora, a disruption that WMP therapy can remarkably reduce.

**Figure 4:** Summary of Taxonomic Analysis Across Groups

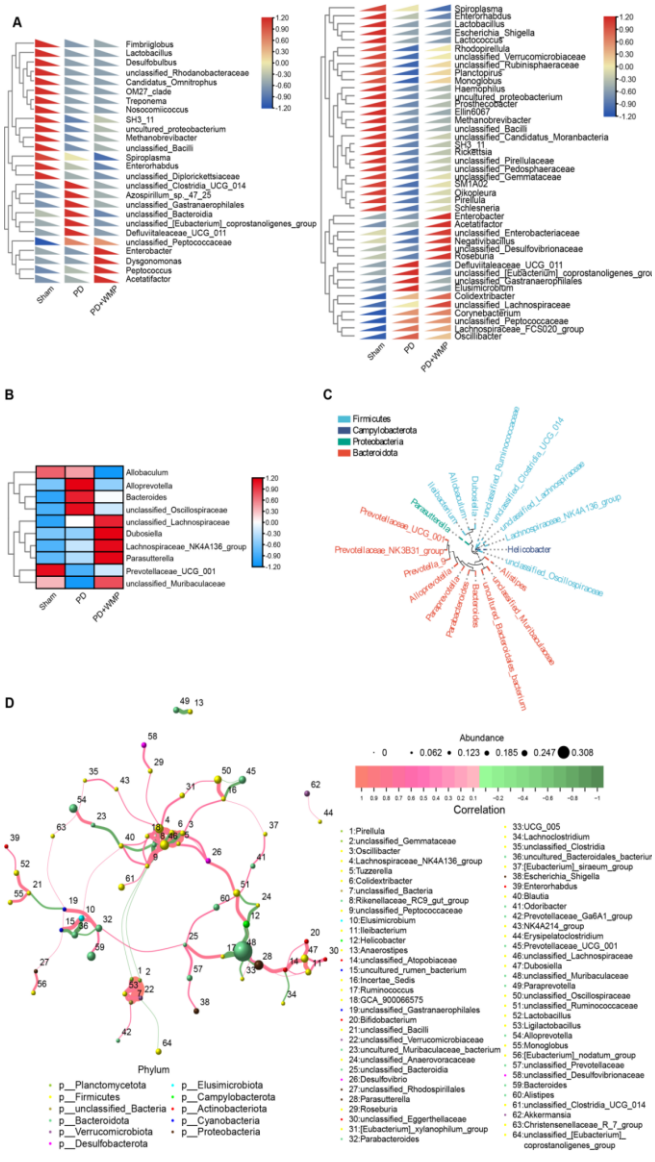


(A) Overview of OTUs distribution in each sample. The visualization captures the diversity and distribution of Operational Taxonomic Units (OTUs) within the studied samples. (B) Non-Metric Multi-Dimensional Scaling (NMDS) Analysis. This graph plots various samples as points, with distinct colors denoting different experimental groups. The spatial separation between points reflects their dissimilarities, with a stress value below 0.2 indicating reliable NMDS analysis. Closer proximity among samples suggests greater similarity. (C) Genus-Level Gut Microbiota Composition. This part displays the relative abundances of gut microbiota across the three groups, with the x-axis labeling the samples and the y-axis showing the percentage of relative abundance. The color-coded stacked bars represent the ten most abundant taxa at the genus level for each sample, illustrating the diversity within the microbial communities. (D) Ternary Phase Diagram Analysis. This analysis utilizes a triangle to represent the relative abundance of species within each sample, where each angle symbolizes a distinct group, and the edges quantify the species' relative abundances for the samples denoted by corresponding colors. The legend identifies the five genera with the highest abundance, represented by colored circles. (E) Graph-Based Phylogenetic Analysis. This section involves analyzing the phylogenetic relationships between the microbial taxa identified in the study. (F) FUNGuild Functional Prediction. This forecasts the functional capabilities of the microbiota, indicating the potential metabolic processes influenced by the different treatments, as observed in the study groups.

## Identification of Gut Flora Genus Modified by Wumei Pill in PD Model

Important genera that distinguished across groups were found by further analysis using ANOVA as well as Rank sum tests. Interestingly, the PD group had considerably higher prevalences of unclassified_Gastranaerophilales, unclassified_[Eubacterium]_coprostanoligenes_group, as well as Defluviitaleaceae_UCG_011. On the other hand, Enterobacter as well as Acetatifactor levels in the PD-Wumei group were significantly higher Figure 5A. Unclassified_Lachnospiraceae, Dubosiella, Lachnospiraceae_NK4A136_group, as well as Parasutterella were significantly abundant in the PD-Wumei one, whereas Alloprevotella, Bacteroides, as well as unclassified_Oscillospiraceae showed noteworthy enrichment in the PD group, according to further genus abundance analysis. Interestingly, the unclassified_Muribaculaceae, which declined in the PD one, increased inversely in the PD-Wumei as well as Sham groups, suggesting it plays a crucial part in PD and that Wumei therapy restores it Figure 5B. Additionally, taxa that were highlighted for their categorical significance included unclassified_Oscillospiraceae, unclassified_Lachnospiraceae, Dubosiella, as well as Lachnospiraceae_NK4A136_group (classified under Firmicutes), as well as Bacteroides as well as unclassified_Muribaculaceae (grouped under Bacteroidota), Alloprevotella, as well as Paraprevotella Figure 5C. A complicated interaction network influenced by Wumei treatment was indicated by the correlation network analysis, which showed interconnections among NO.4 (Lachnospiraceae_NK4A136_group), NO.28 (Parasutterella), NO.46 (unclassified_Lachnospiraceae), NO.48 (unclassified_Muribaculaceae), as well as NO.47 (Dubosiella), all of which were primarily found in the PD-Wumei group Figure 5D.

**Figure 5:** Variation and Prevalence of Gut Microbiota

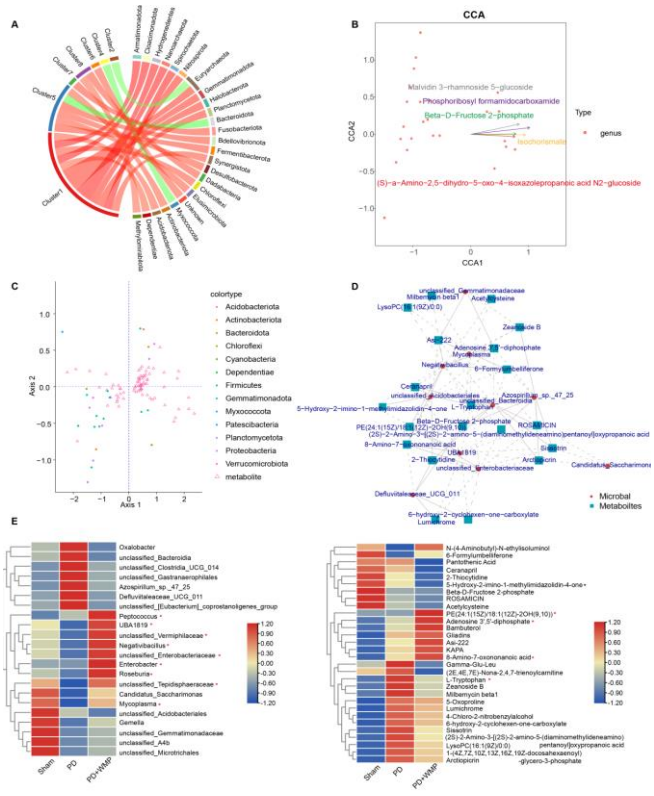



(A) Depiction of differences among samples using ANOVA (left) and Rank sum test (right) analyses. (B) Heatmap showcasing the variations in relative abundance of gut microbiota at the genus level across different groups. (C) Analysis of phylogenetic trees to illustrate changes in gut microbiota composition among the three groups. (D) Analysis of the correlation network. The magnitude of each dot and its connecting lines signify the relative abundance of the microbiota.

### Integrated Metabolomic and Microbiota Analysis

We conducted module-to-module studies between metabolites and microbiotas in an attempt to better understand the processes behind the WMP's impact on gut microbiome in PD (Figure 6A). To identify core components, this was supplemented by coinertia analysis, canonical correspondence analysis, as well as a metabolite-genus network method (Figure 6B-D). A heatmap depiction of the relationships between certain metabolites and microbiotas was then created (Fig 6E). According to our research, possible targets impacted by Wumei therapy included Peptococcus, unclassified_Vermiphilaceae, Negativibacillus, unclassified Enterobacteriaceae, Enterobacter, Roseburia, unclassified_Tepidisphaeraceae, as well as Mycoplasma. Adenosine 3',5'-diphosphate, 8-Amino-7-oxononanoic acid, as well as PE (24:1(15Z)/18:1(12Z)-2OH (9,10) were among the metabolites that were markedly increased in the PD-Wumei one. L-tryptophan levels were notably lower in the PD-Wumei group but higher in the PD one (Figure 6E), indicating the effect of WMP therapy on certain metabolic pathways and microbiota.

**Figure 6:** Connective Analysis of Gut Microbiota and Metabolism




(A) Metabolite-Microbe Chordogram: This plot visualizes the connections between metabolites (left semi-circle) and microorganisms (right semi-circle). Each chord represents a significant correlation between a metabolite and a microorganism, with red indicating positive correlation and green indicating negative correlation. The chord's width signifies the frequency count associated with the metabolite or microorganism. (B) Canonical Correspondence Analysis (CCA): This graphical representation plots microorganisms as points and metabolites as arrows, with the arrow lengths scaled proportionally for clarity, showcasing only the top five metabolites by arrow length. The angle between a microorganism point and an arrow indicates the type of correlation with the metabolite: acute angles suggest positive correlations, obtuse angles suggest negative correlations, and right angles imply no significant relationship. (C) Coinertia Analysis: Circles represent microorganisms (at the genus level), with varying colors denoting different phyla (the first 15 phyla are labeled by name, with others collectively termed '*_other'). Triangles represent metabolites, illustrating the intricate interactions within the microbial-metabolite network. (D) Metabolite-Genus Network: This network diagram illustrates the relationships between specific genera and metabolites, highlighting key interactions within the gut microbiome and metabolic pathways. (E) Heatmap of Hub Metabolites and Microorganisms: This heatmap displays the association between hub metabolites and microorganisms, with the intensity of the color indicating the strength of association, facilitating the identification of significant correlations within the PD-Wumei group.

## DISCUSSION

PD remains a formidable challenge to treat, as current therapies are limited in their ability to slow or halt disease progression Armstrong et al. (2020), Chen et al. (2020), Patel et al. (2020). Furthermore, prolonged use of dopamine replacement treatment may result in adverse consequences or decreased effectiveness Armstrong et al. (2020), Chen et al. (2020), Patel et al. (2020), Group et al. (2014). In PD, dopaminergic neurons in the substantia nigra degenerate, as well as the surviving neurons accumulate α-synuclein-enriched Lewy bodies and neurites. In a 6-hydroxydopamine-induced PD mouse model, our research used a range of methods, like immunohistochemistry, WB, as well as ELISA tests Figure 3A, B, and C, which together verified WMP shield dopaminergic neurons from harm. WMP's promise as a PD treatment and prevention tool is further supported by behavioral assessments Figure 2.

The pathophysiology of PD is remarkably affected by dysbiosis of the gut microbiota, which features a decrease in anti-inflammatory bacteria as well as a rise in pro-inflammatory ones Tansey et al. (2022), Pajares et al. (2020), Marogianni et al. (2020). Significant changes in microbial species, genes, and inflammatory pathways have been found in PD patients, based on a meta-analysis of more than 2000 samples Abdi et al. (2022), Nie et al. (2022). Roseburia, an anti-inflammatory bacterium, was found to be more prevalent in the PD+WMP group, according to our combined study of gut microbiota as well as brain tissue metabolomics (Fig 6E). This result is in line with international research that indicates PD

patients have lower levels of roseburia Nishiwaki et al. (2020). It has been demonstrated that PD reduces the levels of roseburia, a butyrate-producing plant, as well as other short-chain fatty acids (SCFAs) such acetate as well as propionate Morrison et al. (2016), Unger et al. (2016), Lu et al. (2016). Another SCFA-producing family that has been suggested to decline in PD patients, Prevotellaceae_UCG_001, similarly shown recovery in the PD+WMP group Figure 5B Scheperjans et al. (2015), Qin et al. (2010).

The gut-brain axis, a complex communication network between the gastrointestinal (GI) tract as well as the central nervous system, has garnered increasing attention in recent years for its significant role in neurodegenerative diseases. This two-way communication depends on gut microbiota, which also affects behavior, neurotransmission, and brain development. It may even have a role in the genesis and course of disorders like PD Zhang et al. (2021). According to clinical findings, among other changes, PD patients commonly exhibit dysbiosis of the gut microbiota as well as variations in microbial metabolites Sun et al. (2018), Zheng et al. (2021). Prevotellaceae were much lower in the intestines of PD patients than in healthy controls, according to research by Cheperjans et al., and there was a correlation between the quantity of Enterobacteriaceae and PD motor symptoms Grenham et al. (2011). Chinese PD patients' fecal microbiomes were examined by Qian et al., who found an enrichment of particular bacteria as well as associations between particular bacterial genera and cognitive deficits and illness duration Braak et al. (2003). Fecal microbiota transplantation may reduce intestinal and neurological inflammation caused by TLR4/TBK1/NF-ϰB/TNF-α, reduce microglia as well as astrocyte activation, restore SCFAs, as well as increase DA and 5-HT levels in PD mice, potentially preventing disease and improving symptoms Sun et al. (2018). It has been demonstrated to decrease pro-inflammatory factors and reduce intestinal inflammation via the NF-ϰB and MAPK pathways Lu et al. (2022). Additionally, Wumen Pill can elevate the gut flora in rat models of diarrhea-predominant irritable bowel syndrome Lu et al. (2024), change the microbial landscape in obese type 2 diabetes patients Ding et al. (2022), and improve the diversity of intestinal microbiota in obese mice toward a healthier composition Sun et al. (2024).

This research observed a rise in Bacteroides in the PD model, which is known to induce TNF-α secretion via LPS Lin et al. (2019). Treatment with WMP led to a decrease in Bacteroides abundance Figure 5B. Similarly, Azospirillum_sp._47_25, another bacterium linked to inflammation Yu et al. (2023), showed a similar reduction under WMP treatment. Notably, Bambuterol, an anti-inflammatory metabolite, was found to be elevated in the PD+WMP group Figure 6E, further underscoring WMP's role in elevating anti-inflammatory genera as well as mitigating pro-inflammatory ones.

Correlation analysis of the metabolomic with microbiome data revealed key differences, such as a decrease in Azospirillum_sp._47_25, unclassified_Bacteroidia, as well as unclassified_Acidobacteriales in the WMP-treated group, compared to their higher abundance in the PD model Figure 6E. L-Tryptophan, a significant metabolite, was found to be elevated in the PD model but decreased in the PD+WMP group Figure 6E, suggesting that WMP may modulate the kynurenine pathway, which is a major metabolic route for L-Tryptophan.

Coprostanoligene levels were much lower in the control as well as WMP treatment groups and significantly higher in the PD one Figure 6E, suggesting a possible impact on PD risk as well as a function in cholesterol metabolism. The significance of gut microbiota in PD pathophysiology as well as therapy is highlighted by our findings, which are consistent with prior research showing an inverse connection between certain probiotic genera and PD symptoms.

WMP protects dopaminergic neurons and alters the makeup of the gut microbiota, making it a possible therapy option for Parkinson's disease. This work opens up new therapy options centered on gut microbiota modulation by highlighting the significance of the gut-brain axis in the management of PD as well as proposing a unique therapeutic approach.

## CONCLUSION

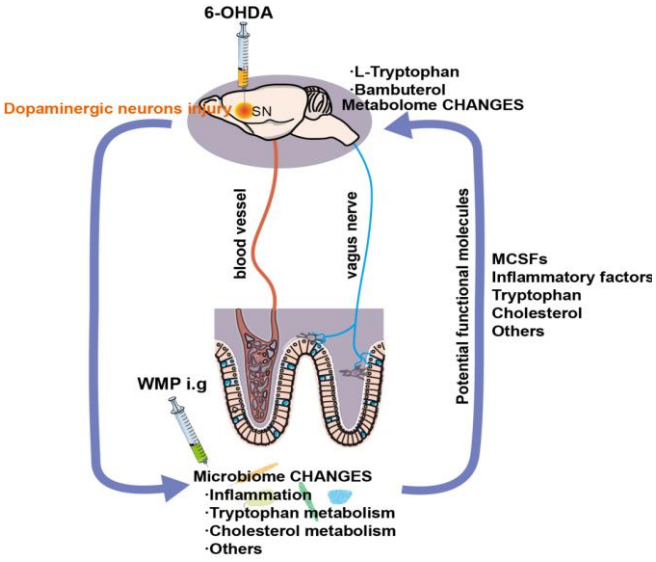
The induction of dopaminergic neuron damage by 6-OHDA disrupts the gut microbiome via the gut-brain axis, as illustrated in the summarized diagram Figure. 7. This results in variations in the bacterial genera involved in serotonin as well as cholesterol metabolism, including Bacteroides, Azospirillum_sp.47_25, unclassified_Bacteroidia, as well as unclassified [Eubacterium]_coprostanoligenes_group, as well as an increased presence of pro-inflammatory bacteria.

The anti-inflammatory genus Roseburia is becoming less abundant at the same time as this harm. However, Wumen Pill therapy successfully reverses these negative alterations, returning the bacterial species indicated above to their typical levels.

At the same time, the anti-inflammatory metabolite Bambuterol is elevated in brain tissues and the substrate of the kynurenine pathway, L-tryptophan, is normalized in the WMP-treated group.

Similarly, following WMP therapy, the consequences of dopaminergic neuron destruction as well as changes in dopamine levels tend to reverse to those seen in the sham-operated group. Additionally, behavioural tests suggest that the model group's motor dysfunctions have significantly improved, demonstrating the therapeutic potential of WMP therapy.

**Figure 7:** Model Diagram: The PD-Brain-Gut-Microbiome Axis and WMP Intervention




## DECLARATIONS

### Data availability

The datasets analysed during the current research are attained from the corresponding author on reasonable request.

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### Author Contributions

The research's conceptualization, methods, as well as manuscript writing were overseen by Zuowen Zhang and Jie Huang, who also carried out Western Blot, immunohistochemistry, and behavioral testing. Lan Shen helped with the behavioral testing, while Yinyou Bai was in charge of the data analysis for the microbiota. Shishuang Li concentrated on the ELISA test. As the corresponding author, Shumei Wang gave guidance and oversaw the manuscript's improvement.

### Conflict of Interest

The authors declare no conflicts of interest.

### Ethics statements

The ethical clearance for this investigation was granted by the Animal Experiment Ethics Committee at Kunming Medical University (Approval Code: KMMU20231408), with the approval dated [14-08-2023]. The methodologies employed throughout this study adhered rigorously to the ethical standards and guidelines outlined in the Guide for the Care and Use of Laboratory Animals (8th edition), issued by the National Institutes of Health (NIH). All animal procedures were conducted with the utmost regard for ethical and compassionate treatment, in full compliance with both national and international animal welfare standards.

### Patient consent for publication

Not applicable.

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