# ORIGINAL RESEARCH Gut-Brain Axis-Based Polygala Tenuifolia and

# Magnolia Officinalis Improve D-gal-Induced Cognitive Impairment in Mice Through cAMP and NF-κB Signaling Pathways

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Purpose: Polygala tenuifolia Willd. (PT) is commonly used to address cognitive impairment (CI), while Magnolia officinalis Rehd. et Wils (MO) is often prescribed for gastrointestinal issues as well as CI. This study seeks to explore the impacts and mechanisms behind the combined therapy of PT and MO (PM) in treating CI, based on the concept of the gut-brain axis.

Methods: The characteristic components of PT, MO, and PM were identified using ultra-high performance liquid chromatographytandem triple quadrupole mass Spectrometry (UPLC-MS/MS). A mouse model was established by D-gal induction, and the effects of PT, MO, and PM on CI were evaluated through behavioral tests, pathological staining, and Enzyme-Linked Immunosorbent Assay (ELISA). Subsequently, network pharmacology was used to analyze the potential mechanisms by which PM improves CI, followed by validation through Western blotting (WB), traditional Chinese medicine (TEM), Immunofluorescence (IF), and 16S rRNA.

Results: PT, MO, and PM can each alleviate cognitive decline and neuropathological damage in D-gal mice to varying degrees, reduce the expression of pro-inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IFN- $\gamma$ , LPS) in serum or hippocampal tissue, and increase SOD and GSH levels. Network pharmacology analysis and molecular experiments confirmed that PM upregulates the expression of tight junction s (TJs), enhances the expression of proteins in the cAMP pathway, and inhibits p-NF-kB-p65 expression. PM reverses D-gal-induced gut microbiota dysbiosis, increases the abundance of SCFA-producing bacteria, and decreases the abundance of LPSproducing bacteria.

Conclusion: PM alleviates CI by reducing inflammation and oxidative stress, protecting the blood-brain barrier (BBB) and intestinal barrier, inhibiting the NF-κB pathway, activating the cAMP pathway, and regulating gut microbiota.

**Keywords:** polygala tenuifolia, magnolia officinalis, cognitive impairment, cAMP Signaling Pathway, NF-κB Signaling Pathway, gut microbiota

#### Introduction

CI denotes one or several deficits in a person's cognitive abilities, which may include memory, attention, executive functioning, language, and visuospatial skills.<sup>1-3</sup> The range of severity includes mild cognitive impairment, in addition to moderate and severe forms of dementia.<sup>4,5</sup> Currently, more than 55 million individuals worldwide are affected by dementia, with around 10 million new instances recognized annually.<sup>6</sup> The effects of dementia are significant for both the individuals diagnosed and their families, posing considerable challenges to the sustainability of elderly care and healthcare services.<sup>7</sup>

CI can arise from a multitude of factors, notably including aging, Alzheimer's disease (AD), and depression, with AD being one of the most prevalent forms of CI.<sup>8-12</sup> Recent scholarly research indicates that an imbalance in gut microbiota,

#### **Graphical Abstract**



referred to as dysbiosis, could play a role in the onset of CI.<sup>13</sup> Animal studies utilizing mouse models have shown that when gut microbiota from Alzheimer's mice are transplanted or when probiotics are administered, there are observable changes in amyloid beta ( $A\beta$ ) pathology.<sup>14,15</sup> This finding reinforces the idea that gut microbiota might significantly influence the fundamental processes associated with AD. In addition, numerous case-control studies have noted changes in the gut microbiota composition in people diagnosed with mild cognitive impairment and AD.<sup>16</sup> The gut microbiome serves as an essential component of the gut-brain communication pathway, which is a sophisticated network facilitating interactions between the brain and the digestive system.<sup>17</sup> Through various pathways and mechanisms, the gut microbiota communicates with the brain, which can significantly influence the overall health of the host.<sup>18–20</sup> Recent studies have highlighted the significance of the gut-brain connection in controlling various physiological and pathological mechanisms, including mood regulation, cognitive functionality, and inflammatory responses.<sup>21</sup> The functioning of this axis is influenced by the gut microbiota through the production of particular metabolites, including SCFAs, secondary bile acids, and tryptophan-derived metabolites. These metabolites may, in turn, have profound effects on cognitive functions and emotional states.<sup>22</sup> These results collectively indicate a significant connection between gut microbiota and cognitive impairment, suggesting that gut microbiota could be pivotal in the development and advancement of cognitive impairment, potentially facilitated by the gut-brain axis mechanisms.

At present, there is a shortage of effective treatment options for CI, highlighting the importance of early diagnosis and timely intervention to enhance prognosis and overall quality of life.<sup>23–25</sup> Although there have been numerous studies on drugs for treating cognitive impairment, commonly used drugs such as memantine and donepezil have shown limited efficacy in improving cognitive abilities.<sup>26,27</sup> Traditional Chinese medicine offers multiple target effects and has few side effects,<sup>28</sup> which makes it a frequently utilized method for both preventing and treating cognitive impairment, including the use of ginkgo biloba extract<sup>29</sup> and huperzine A.<sup>30</sup> PT refers to the dried root of *Polygala tenuifolia* Willd. From the Polygalaceae family, and MO refers to the dried root bark and branch bark of *Magnolia Officinalis* Rehd.et Wils from the

Magnoliaceae family. Both PT and MO are initially recorded in "Shennong's Materia Medica", where PT is noted for its abilities to soothe, enhance cognition, relieve phlegm, and act as an antidepressant. This indicates that PT has a positive impact on cognitive function. Contemporary research has shown that PT provides protective benefits for the central nervous system and has the potential to ease symptoms including memory deficits, insomnia, and neurasthenia.<sup>31</sup> The component of PT, Tenuifolin (TEN),<sup>29</sup> has demonstrated a significant improvement in CI and memory learning, suggesting its potential utility as a therapeutic agent in the treatment of CL<sup>32,33</sup> TEN influences various biological processes, such as hindering the accumulation of A $\beta$  related to neurodegenerative damage, decreasing levels of acetylcholine, lessening neuroinflammatory responses, mitigating oxidative harm to cells, and blocking the apoptosis of neurons.<sup>34–37</sup> MO is known to dry dampness, eliminate phlegm, and alleviate fullness, which is why it is frequently used in traditional Chinese medicine to address gastrointestinal disorders.<sup>38</sup> In traditional Chinese medicine, MO has also been used to regulate qi and blood as well as dredge the meridians. In Traditional Chinese Medicine theory, smooth flow of qi and blood and harmonious meridians are closely associated with the cognitive state of the brain. Recent research has shown that MO has a substantial effect on the nervous system, displaying properties such as anti-epileptic, antidepressant, anti-dementia, and anti-cerebral ischemia activities. The primary active ingredients, magnolol and honokiol, can easily penetrate the BBB.<sup>39–41</sup> Research has shown that MO can improve cognitive impairments in mice with AD by stimulating mitochondrial SIRT3,<sup>42</sup> suggesting its promise as a versatile treatment option for neurodegenerative disorders.

The management of CI using PM has been practiced for many years. An example is the Zhu Li Decoction, which was recorded by the esteemed Tang Dynasty doctor Sun Simiao in "Qianjin Yaofang" and is applied to treat signs like confusion and speech difficulties.<sup>43,44</sup> In a similar vein, the Wuxie Pill mentioned in Wang Tao's "Wai Tai Mi Yao" targets symptoms of agitation and mental confusion, both of which are important indicators of CI.<sup>45</sup> Both formulations contain PT and MO, which has traditionally been used to treat neurological conditions, thus clarifying the traditional application of PM. Previous research has indicated that PM can enhance gastrointestinal motility,<sup>46</sup> promote the growth and proliferation of gastric stromal cells, lower NO levels in the gastric antrum, decrease the expression of VIP,<sup>47</sup> hinder the absorption of glycosides derived from PT while facilitating their elimination,<sup>48</sup> and regulate the gut microbiota and inhibiting the activation of PT with MO not only maintains the sedative and expectorant properties but also amplifies them, resulting in a synergistic effect and minimizing side effects.<sup>50</sup> This research explores the mechanisms and effects of PM in enhancing CI as informed by the gut-brain axis, offering experimental support for the safe and rational application of PM. The graphical abstract illustrates the overall research design and main findings.

#### **Materials and Methods**

#### Chemicals and Reagents

D-gal (Lot No. 2023042601), methanol specifically designated for chromatography (Lot No. 2022032801), and formic acid (Lot No. 2022112001) were procured from Chengdu Cologne Chemical Co., Ltd. (Sichuan, China). Additionally, chromatographic-grade acetonitrile (Lot No. RBXA1H) was sourced from SK Chemicals (Gyeonggi-do, South Korea). 4% paraformaldehyde (Lot No. GP231030112930) and goat serum (Lot No. GA2403012) were both purchased from Wuhan Service Biotechnology Co., Ltd. (Wuhan, China). Nissl solution (Lot No. 0704A24) was obtained from Jiangsu Edison Biotechnology Co., Ltd. (Jiangsu, China). TEN (Lot No. 111849–202,207), Polygalaxanthone III (Lot No. 111850–202,207), 3.6'-diferuloyl sucrose (Lot No. 111848–202,307), and Polygalacic acid (Lot No. 111939–201201) were purchased from the National Institute for Food and Drug Control (Beijing, China). Honokiol (Lot No. MUST-17031102) and Magnolol (Lot No. 202402), IL-1 $\beta$  (Lot No. 202402), IL-6 (Lot No. 202405), IFN- $\gamma$  (Lot No. 202402), LPS (Lot No. 202402), superoxide dismutase (SOD, Lot No. 202402), GSH (Lot No. 202402), cAMP (Lot No. 202405), and SCFA (Lot No. 202405) kits were obtained from Shanghai Fankwei Industrial Co., Ltd. (Shanghai, China). The BCA protein assay kit (Lot No. 037C1500) was sourced from Shanghai EpiZyme Biotechnology Co., Ltd. (Shanghai, China). Occludin (Catalog#272260-1-AP), ZO-1 (Catalog#21773-1-AP), p65 (Catalog#10745-1-AP), and

p-p65 (Catalog#82335-1-AP) were all purchased from Wuhan San Ying Biotechnology Co., Ltd. (Wuhan, China). p-PKA (Catalog#HA721864), PKA (Catalog#ER64618), p-CREB (Catalog#ET7107-93), CREB (Catalog#ET-1601-15), GAPDH (Catalog#ET1601-4), goat anti-rabbit HRP (Catalog#HA1001), and iFluor<sup>™</sup> 488 (Catalog#HA1121) were all obtained from Hangzhou Hua'an Biotechnology Co., Ltd. (Hangzhou, China).

#### Preparation of Plant Materials and Aqueous Extracts

PT (batch number: 221101, report number: 5–273-02-221101) and MO (batch number: 221201, report number: 5–259-02-221201) were acquired from Sichuan Guoqiang Traditional Chinese Medicine Products Co., Ltd. (Sichuan, China). The specifications outlined in the 2020 edition of the Pharmacopoeia of the People's Republic of China are met by both PT and MO.<sup>51</sup> The methodologies for extraction and the ratio of the combined PM were informed by prior research conducted by our team.<sup>48,52</sup> We utilized 50 g of PT alongside 100 g of MO. PT and MO were combined with ten times its volume of distilled water, allowed to soak for 30 minutes, and then brought to a boil. After reaching a boil, the heat was lowered; PT was simmered for one hour, then filtered and concentrated under reduced pressure to produce 50 mL, whereas MO continued to simmer for an additional 15 minutes, was filtered, and concentrated to obtain 100 mL.

Additionally, 50 g of PT was obtained and mixed with 500 mL of distilled water, allowing it to soak for half an hour, brought to a high boil, and then simmered at a lower temperature for 45 minutes. The 100 g of MO that had been previously soaked was merged with the PT infusion, which was heated for 45 minutes, brought to a boil, and then simmered for another 15 minutes. After filtering, the mixture was concentrated under reduced pressure, resulting in a yield of 50 mL. This final solution was then stored separately at  $-80^{\circ}$ C.

#### **Component Analysis**

The methods of analysis were derived from earlier studies conducted by the research team.<sup>49</sup> A 70% methanol solution was utilized to dissolve PT, MO, and PM, followed by filtration through a 0.22  $\mu$ m microporous membrane. An UPLC system (Vanquish, Thermo Scientific, Waltham, MA, USA) was utilized for the analytical procedures. This UPLC system was effectively combined with a triple quadrupole mass spectrometer (TSQ Fortis, Thermo Scientific, Waltham, MA, USA). Notably, the mass spectrometer featured advanced electrospray ionization capabilities. The samples were separated using a C18 column (3×100 mm, 2.6  $\mu$ m) kept at a temperature of 40°C, with a flow rate of 0.2 mL/min and an injection volume of 5  $\mu$ L. The gradient elution profile was designed as follows: 0–5 minutes, 2%-10% B; 5–15 minutes, 10%-30% B; 15–20 minutes, 35%-50% B; 20–25 minutes, 50%-70% B; 25–30 minutes, 70%-95% B; 30–35 minutes, 95% B. In the mass spectrometry process, the setup included positive ionization at 4000 V, negative ionization set at 3500 V, sheath gas pressure at 35 kPa, auxiliary gas pressure at 10 kPa, and a gas temperature maintained at 350°C. For the data collection and analysis, Thermo Scientific Xcalibur (version 4.2) was employed.

#### Animals and Experiments

Eight-week-old male KM mice, weighing between 18 and 22 grams, were obtained from Sibeifu Biotechnology Co., Ltd. (No. 110324231107055163, Beijing, China) and housed in a facility for small animal observation, where humidity was kept at  $65 \pm 5\%$  and temperature was regulated to  $22 \pm 2^{\circ}$ C with 12 h/12 h light-dark cycle. Prior to the commencement of the study, all animal experiments were conducted in accordance with the principles of animal ethics and the Regulations for the Administration of Experimental Animals established by the People's Republic of China. These experiments were reviewed and approved by the Animal Welfare and Ethics Committee of Chengdu University of Traditional Chinese Medicine, with the approval number 2024046.

The mice were randomly split into multiple groups, with each group consisting of 8 individuals: the Control group and the D-gal group, Donepezil group (0.75 mg/kg, designated as Don + D-gal), low-dose PT group (0.25 g/kg, referred to as P L + D-gal), medium-dose PT group (0.5 g/kg, labeled P M + D-gal), high-dose PT group (1 g/kg, denoted P H + D-gal), low-dose MO group (0.5 g/kg, M L + D-gal), medium-dose MO group (1 g/kg, M M + D-gal), high-dose MO group (2 g/kg, M H + D-gal), low-dose PM group (0.25 + 0.5 g/kg, PM L + D-gal), medium-dose PM group (0.5 + 1 g/kg, PM M + D-gal), and high-dose PM group (1 + 2 g/kg, PM H + D-gal). For a duration of 42 consecutive days, all mice, apart from those in the control group, were administered subcutaneous injections of D-gal dissolved in saline at a

dosage of 500 mg/kg·d to create a cognitive impairment model. In contrast, the mice in the control group received an equivalent volume of saline. Based on the human drug dosage, the drug doses for mice were determined using the body surface area conversion method.<sup>51,53</sup> After the D-gal injections, each treatment group was given the respective medications orally for 42 days, whereas the control and D-gal treated mice were provided daily with distilled water at a rate of 10 mL/kg·d.

On the 43rd day, mice underwent anesthesia using a 10% urethane solution. Following this, brain tissues, along with intestinal tissues and their respective contents, were obtained from the mice. A segment of both the brain and intestinal tissues was preserved in a 4% paraformaldehyde solution and subsequently stained using H&E or Nissl staining. The remaining hippocampal tissue was isolated from the brain and preserved in 3% glutaraldehyde for examination via transmission electron microscopy. Additionally, the extra hippocampal tissue, along with intestinal tissue and its contents, was kept at  $-80^{\circ}$ C. The hippocampal tissue was allocated for ELISA and WB studies, whereas the intestinal tissue was designated for Western blot analysis, and the intestinal contents were arranged for 16S rRNA metagenomic assessment. All experimental procedures were carried out following the established protocol (Figure 1).

#### **Behavioral Tests**

The cognitive function in mice was evaluated using the PAT (TechMan Soft, Sichuan, China) and the MWM (WMT-100S, Sichuan, China).<sup>53</sup> Testing with the PAT occurred the day after the mice had undergone adaptive training in the apparatus. The PAT-8 video analysis system systematically captured both the latency for escape and the count of errors made. During the MWM spatial navigation assessment, all mice participated in one day of adaptive training with a visible platform, which was followed by four days of trials that involved a hidden platform in the MWM apparatus, with each trial lasting 60 seconds. On day six, the platform was removed, and a spatial exploration assessment was performed in the MWM. The WMT-100S video imaging analysis system automatically recorded the distance swum in the designated quadrant, the number of platform crossings, and the time spent in the target area.



Figure I Experimental flow diagram.

#### Histological Examination

Following fixation in a solution of 4% paraformaldehyde for a duration of 48 hours, the brain and intestinal samples underwent dehydration, embedding, and sectioning. Sections of mouse brain and intestinal tissue were prepared with a uniform thickness of 5 µm. These sections, which had been embedded in paraffin, underwent staining using the H&E method. Following the staining process, the sections were examined and visualized using a microscope (Sunny, Hong Kong, China). Brain tissue sections were stained using Nissl staining technique and scanned with a digital pathology scanning system (HS6, Zhejiang, China). For IF, the paraffin sections were incubated in an oven set at 60°C for one hour, deparaffinized using a gradient of ethanol, and subjected to antigen retrieval with an EDTA solution. The sections underwent permeabilization using 0.5% Triton for thirty minutes and were subsequently blocked at room temperature with goat serum for one hour. Following this, they were incubated overnight with a primary antibody specific to p-CREB (1:100) and then treated with a secondary antibody (1:200) at room temperature for an additional hour. All sections were then counterstained with DAPI to enable visualization of the nuclei. Fluorescent images were captured employing a fluorescence microscope (Zeiss Axio Imager 2, Germany), and fluorescence intensity was measured utilizing ImageJ software. For TEM, the tissues were subjected to a 3% glutaraldehyde solution, fixed with 1% osmium tetroxide, dehydrated in acetone, and infiltrated with epoxy resin (Epox) 812 over a prolonged period. Once embedding was finalized, ultrathin sections were cut using a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEM-1400-FLASH TEM (JEOL, Japan).

#### **ELISA** Assay

Weigh the brain tissue of the mouse and homogenize it using a 1:10 ratio with physiological saline. After centrifuging, collect the supernatant to measure the protein concentration. Transfer the mouse blood into a centrifuge tube and let it rest at room temperature for a duration of two hours. Following this interval, isolate the serum using high-speed centrifugation at low temperatures. Subsequently, transfer it into a new centrifuge tube and store it at  $-80^{\circ}$ C. Next, assess the levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IFN- $\gamma$ , and LPS in the mouse serum, and evaluate TNF- $\alpha$ , IL-1 $\beta$ , SOD, GSH, cAMP, and SCFA concentrations in the mouse brain tissue following the guidelines provided by the manufacturer's ELISA kits.

#### Network Pharmacology

Drug targets were identified through the utilization of various databases, which include CNKI (https://www.cnki.net/), PubMed (https://pubmed.ncbi.nlm.nih.gov/), Herb (http://herb.ac.cn/), SwissTargetPrediction (http://www.swisstargetpre diction.ch/), TCMSP (https://old.tcmsp-e.com/), and BATMAN-TCM (http://bionet.ncpsb.org.cn/batman-tcm/index.php/ Home/Index/index). Additionally, genes that are linked to CI were discerned using the GeneCards (https://www. genecards.org/) and OMIM databases (https://omim.org/), enabling a more focused investigation into the genetic factors contributing to CI. To further refine the analysis, the intersection between the potential drug targets for MO and PT with the CI-related genes was established using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/). This crossing enabled the recognition of common targets that could be involved in the therapeutic uses of MO and PT within CI contexts. The analysis was deepened by utilizing the STRING 12.0 database (https://string-db.org/) to evaluate potential therapeutic targets, from which a protein-protein interaction (PPI) network was developed using Cytoscape 3.7.2. This network serves as a visual representation of the interactions among identified proteins, thus providing insight into their functional relationships. For further visualization and analytical depth, additional tools were employed, including a bioinformatics online analysis and visualization cloud platform (https://www.bioinformatics.com.cn/). Through this platform, KEGG pathway enrichment bubble charts and GO functional enrichment bar charts were generated, highlighting significant biological pathways and functions associated with the therapeutic actions of PT. The foremost 20 pathways, selected according to their P-values, were deliberately identified to highlight essential pathways related to the treatment of CI. Ultimately, the tool Cytoscape 3.7.2 was employed to create an extensive network diagram illustrating the relationships among drugs, diseases, key targets, and important pathways. This diagram not only illustrates the interrelations but also contributes to a deeper understanding of the therapeutic landscape for cognitive impairment.

## 16S rRNA Sequencing of Gut Microbiota

Measure the total DNA extracted with a Nanodrop spectrophotometer. Employ PCR to amplify the variable rRNA gene regions or particular gene fragments, and subsequently purify the resulting PCR products with Vazyme VAHTS<sup>™</sup> DNA Clean Beads, eluting them in buffer. Use the Illumina TruSeq Nano DNA LT Library Prep Kit to generate the sequencing library. Perform paired-end sequencing on the MiSeq sequencer by employing the MiSeq Reagent Kit V3 (600 cycles). Implement sequence denoising or OTU clustering per the QIIME2 DADA2 analysis protocol. Use QIIME2 (version 2019.4) software for species annotation.

### Western Blot Analysis

The samples underwent lysis using RIPA buffer, after which the supernatant was obtained through centrifugation. The concentration of total protein in the samples was measured utilizing the BCA protein assay method. To separate the target proteins, SDS-PAGE was utilized, and these proteins were then transferred onto PVDF membranes. Primary antibodies, including Occludin (1:5000), ZO-1 (1:10,000), NF- $\kappa$ B-p65 (1:1000), p-NF- $\kappa$ B-p65 (1:2000), PKA (1:1000), p-PKA (1:1000), CREB (1:1000), p-CREB (1:1000), and GAPDH (1:5000), utilized to incubate the membranes overnight at 4° C. On the following day, the membranes underwent treatment with a secondary antibody diluted to 1:50,000. Visualization and semi-quantification of the bands were performed through a chemiluminescence imaging and analysis system (GelView 6000Plus, Guangzhou, China).

### Statistical Analysis

The results are displayed as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism 8 software. To compare the groups, a one-way analysis of variance (ANOVA) was executed, alongside suitable post hoc tests. A p-value of less than 0.05 was considered statistically significant.

# Result

## Determination of Characteristic Components in PT, MO, and PM

The findings illustrate a comparison of mass-to-charge ratios alongside standard samples (Figure 2A). In PT, components such as TEN, Polygalaxanthone III, 3.6'-disinapoyl sucrose, and Polygalacic acid were identified (Figure 2B). In MO, the components Honokiol and Magnolol were observed (Figure 2C). Additionally, these specific components were also found in the PM combination (Figure 2D).

# Effects of PT, MO, and PM on Cognitive Dysfunction and Neuropathology Induced by D-gal in Mice

The findings of the PAT study demonstrated that, when compared to the D-gal group, every dosage category of PT and PM, in conjunction with the M M and M H groups, significantly prolonged the escape latency while reducing the number of errors (P < 0.05 or P < 0.01, Figure 3A and B). In addition, compared with each dose group of MO, PM significantly increased the escape latency of mice (P < 0.01, Figure 3A and B). When compared with the P M and P H groups, both the PM M and PM H groups significantly enhanced the escape latency of mice (P < 0.01, Figure 3A and B).

When compared to mice treated with D-gal, there was a notable decrease in escape latency during the MWM navigation test for the P M, P H, M H, and all PM dosage groups (P < 0.05 or P < 0.01, Figure 3C). In the spatial exploration phase of the MWM, there was a significant enhancement in the total swimming distance within the target quadrant for the P M, P H, and all PM dosage groups (P < 0.01, Figure 3D). Compared with each corresponding dose group of PT and MO, PM significantly increased the swimming distance of mice in the target quadrant (P < 0.05 or P < 0.01, Figure 3D). Furthermore, there was a notable increase in the frequency of platform crossings observed in the P M, P H, M H, and all PM treatment groups (P < 0.01, Figure 3E). Compared with P M, M L, and M M, the corresponding dose of PM significantly increased the number of times mice crossed the target quadrant (P < 0.05 or P < 0.01, Figure 3E). In the P M, P H, M H, and all PM treatment groups, a lengthened time was spent in the target quadrant (P < 0.05 or P < 0.01, Figure 3E). Compared with P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P L, P H, and each dose group of MO, PM significantly increased the P M,



Figure 2 UPLC-MS/MS Examination of PT, MO, and PM. (A) Typical constituents in the standard mixture of PM, (B) PT sample, (C) MO sample, (D) sample of PM.

dwell time of mice in the target quadrant (P < 0.05 or P < 0.01, Figure 3F). It is noteworthy that PM treatment yielded better behavioral results than PT or MO treatments (Table 1).

Furthermore, PT, MO, and PM interventions significantly mitigated neuropathological alterations in the hippocampus of aging mice, which included neuronal cytoplasmic shrinkage, disordered arrangement, and a decrease in neuronal counts (Figure 4A). In comparison to the D-gal group, administering PT, MO, and all PM dosages resulted in a significant increase in neuronal cells within the CA1 region (P < 0.05 or P < 0.01, Figure 4B), while the PM H group exposed a substantial enhancement of neuronal numbers in the CA3 region (P < 0.05, Figure 4C). Nissl staining results showed that the Nissl+ neuron rate in the D-gal group was significantly reduced compared to the Control group (P < 0.01, Figure 4D–F). In the CA1 region, PT, MO, and PM at all doses significantly restored the Nissl+ neuron rate reduced by D-gal (P < 0.05 or P < 0.01, Figure 4E). Compared with P M, P H, M M, and M H, the corresponding doses of PM significantly increased the Nissl+ neuron rate in the CA1 region of mice (P < 0.05 or P < 0.01, Figure 4F). Compared with P M, P H, M M, and M H, the corresponding doses of PM significantly increased the Nissl+ neuron rate in the CA1 region of mice (P < 0.05 or P < 0.01, Figure 4F). Compared with M M and M H, the corresponding dose groups of PM significantly enhanced the Nissl+ neuron rate in the CA3 region (P < 0.05 or P < 0.01, Figure 4F). The Nissl staining results further demonstrated the neuroprotective effect of PM. Notably, PM exhibited superior recovery of neuronal pathology compared to either PT or MO alone (Table 1). These results indicate that PT, MO, and PM may improve cognitive impairments in D-gal-made models by reducing neuronal damage.



Figure 3 PT, MO, and PM Alleviate Cognitive Impairments Induced by D-gal. PAT results showing (**A**) escape latency and (**B**) number of errors. MWM results showing (**C**) escape latency, (**D**) accumulated swimming distance in target quadrant of the spatial exploration test, (**E**) times of crossing the target quadrant, (**F**) time in target quadrant, and (**G**) representative swimming trajectories. n = 8.  $^{#}P < 0.05$ ,  $^{##}P < 0.01$  vs Control group;  $^{*}P < 0.05$ ,  $^{**P} < 0.01$  vs D-gal group,  $^{\circ}P < 0.05$ ,  $^{\circ\circ}P < 0.01$  vs PT group,  $^{\Delta}P < 0.05$ ,  $^{\Delta\Delta}P < 0.01$  vs MO group.

Group		Significant Difference												
Metric			Control	D-gal	Don+ D-gal	P L+ D-gal	P M+ D-gal	P H+ D-gal	M L+ D-gal	M M+ D-gal	M H+ D-gal	PM L+ D-gal	PM M+ D-gal	PM H+ D-gal
PAT	Escape latency			#	**	**	**	**		**	**	**,ΔΔ	**,○,∆∆	**,00,44
	Error times			#	**	*	**	**		**	**	**	**,Δ	**
MWM	Escape latency			###	**		*	**			*	*	**	**
	Accumulated swimming			###	**		**	**				**,00,44	**,0,\D	**,००,∆∆
	distance in target													
	quadrant													
	Times of crossing the			###	**		*	**			*	**,Δ	**,0,\D	**
	target quadrant													
	Time in target quadrant			###	**		*	**			*	**,0,44	**,\DD	**,0,ΔΔ
H&E staining	Neuron ratio in CAI			###	**	**	**	**	*	**	**	**	**	**
Neuron ratio in CA3			###	*									*	
Nissl staining	Nissl staining Nissl+ ratio in CAI Nissl+ ratio in CA3			###	**	**	**	**	*	**	**	**	**,0,\D	**,००,∆∆
				###	**	*	*	**				**	**,Δ	**,\DD
ELISA	Hippocampus	TNF-α		###	**			**			*			**
		IL-Iβ		##	*			*						**
		SOD		##	*									**,○,∆
		GSH		###										**,00,44
	Serum	TNF-α		###	*			*						*
		IFN-γ		###	**			**						**,ΔΔ
		IL-Iβ		###	**			**			*			**,Δ
		IL-6		###	**			**			*			**,00,44
		LPS		#	*									*,∘,∆

Table I Significant Differences in PT, MO, and PM in Behavioral Tests, Pathological Staining, and ELISA

Notes: <sup>#</sup>P < 0.05, <sup>##</sup>P < 0.01 vs Control group; <sup>\*</sup>P < 0.05, <sup>\*\*</sup>P < 0.01 vs D-gal group, <sup>°</sup>P < 0.05, <sup>°°</sup>P < 0.01 vs PT group, <sup>Δ</sup>P < 0.05, <sup>ΔΔ</sup>P < 0.01 vs MO group.



**Figure 4** PT, MO, and PM Alleviate D-gal-Induced Neuropathological Damage. (A–C) Representative images and quantitative analysis of HE staining in the hippocampal CA1 and CA3 regions (scale bar: 200  $\mu$ m and 50  $\mu$ m). (D–F) Representative images and quantitative analysis of Nissl staining in the hippocampal CA1 and CA3 regions. Black arrows indicate shrunken neurons (scale bar: 500  $\mu$ m and 50  $\mu$ m). n = 5. <sup>##</sup>*P* < 0.01 vs Control group; \**P* < 0.05, \*\**P* < 0.01 vs D-gal group, °*P* < 0.05, °°*P* < 0.01 vs PT group,  $^{\Delta}P$  < 0.01 vs MO group.

# PT, MO, and PM Treatments Alleviated Inflammation and Oxidative Stress Induced by D-gal

Neuropathological research involving mice suggests that PM is effective in diminishing neuronal damage, with PM H showing the most notable enhancement. Considering that oxidative stress and inflammation contribute to neuronal damage, we investigated the effects of P H, M H, and P M H on markers of oxidative stress and pro-inflammatory factors in serum and brain tissue. In the hippocampal area, the administration of P H, M H, and PM H resulted in a notable decrease in TNF- $\alpha$  levels compared to the D-gal group (P < 0.05 or P < 0.01, Figure 5A). Additionally, both P H and PM H induced a significant reduction in IL-1 $\beta$  level (P < 0.05 or P < 0.01, Figure 5B), while PM H specifically increased the levels of SOD and GSH (P < 0.01, Figure 5C and D). Additionally, compared with P H and M H, PM H significantly increased the levels of SOD and GSH in the hippocampus of mice (P < 0.05 or P < 0.01, Figure 5C and D). Serum analysis revealed that P H and PM H significantly reduced levels of TNF- $\alpha$  and IFN- $\gamma$  as well (P < 0.05 or P < 0.01, Figure 5E and F). Compared with M H, PM H significantly decreased the level of IFN -  $\gamma$  (P < 0.01, Figure 5G and H), with PM H also significantly decreasing the concentration of LPS (P < 0.05, Figure 5I). Compared with M H, PM H significantly reduced the levels of IL - 1 $\beta$ , IL - 6 and LPS (P < 0.05 or P < 0.01, Figure 5G–I). Compared with P H, PM H significantly reduced the levels of IL - 6 and LPS (P < 0.05 or P < 0.01, Figure 5H and I). These results indicate that, compared with P H and M H, PM H exhibits more potent anti - inflammatory and antioxidant effects (Table 1).

#### Network Pharmacology Analysis

Using the Venny 2.1 tool to analyze the intersection between pulmonary PM and CI, A total of 232 overlapping targets were identified, which could act as possible therapeutic options for tackling CI within the framework of PM (Figure 6A). This finding underscores the relevance of these targets in the pursuit of effective treatments. Furthermore, in our examination of the protein-protein interaction (PPI) network, we noted that nodes that are larger in size and display darker colors correspond to higher degree values, thus emphasizing their importance within the overall network of interactions. Our comprehensive data analysis indicated that among all the identified nodes, the five core nodes exhibiting the highest degree values were INS, TNF, PPKACA, CASP3, and IL1B (Figure 6B). These core nodes are critical for







Figure 6 Network pharmacology analysis of PM. (A) Venn diagram, (B) PM's medicinal effects on key target protein-protein interaction (PPI) network diagram, (C) GO enrichment analysis, (D) KEGG enrichment analysis, (E) PM for CI disease key target protein-key pathway network diagram, (F) cAMP and NF-κB KEGG pathway diagrams. The red box represents the signaling pathway validated in our study.

understanding the biochemical pathways at play and could be central to developing targeted interventions for managing cognitive impairment associated with pulmonary metastasis.

The data was subsequently entered into the Metascape database to conduct an enrichment analysis, resulting in a total of 2730 Gene Ontology (GO) annotations. The total includes 2269 biological processes (BP), 189 cellular components,<sup>54</sup> and 272 molecular functions (MF). For each category, the ten highest-ranked entries were chosen based on their P-value rankings and visualized using the online tool available on the Microbioinformatics website. In terms of BP, the primary functions that were identified included the regulation of membrane potential, system process regulation, chemical synapse function, and the processes of synaptic signaling. In the context of CC, the primary components recognized included the synaptic membrane, the complex of monatomic ion channels, the transport complex, and the transmembrane transporter assembly. Within the MF category, the key functionalities encompassed activities related to monatomic ion transmembrane transporters, transmembrane transport for inorganic substances, monatomic cation transmembrane transport encomplex, and functions associated with monatomic ion channels (Figure 6C).

The KEGG analysis proceeded to select the twenty pathways that displayed the smallest P-values and the highest counts of gene enrichment for the purposes of visualization, as illustrated (Figure 6D). In this representation, pathways characterized by more intense red colors indicate the presence of smaller P-values, elevated gene enrichment counts, and a correspondingly stronger significance of the pathways themselves. The visual data suggests that the therapeutic impact of PM on CI may be closely linked to certain biological pathways, particularly the cAMP signaling pathway and the NF- $\kappa$ B signaling pathway (Figure 6F). To further elucidate these relationships, we imported the top twenty pathways along with their relevant targets into Cytoscape 3.7.2 software. This action allowed us to construct a comprehensive network diagram that highlights the interconnections among the disease, significant biological targets, and critical pathways (Figure 6E). In this network, the red nodes signify the crucial biological targets, while the blue nodes depict the important pathways, and the yellow node designates the disease itself. In total, the diagram encompasses 149 nodes and 607 edges, underscoring that the mechanism through which PM operates against CI involves a complex interplay of various components, biological targets, and significant pathways.

#### PM Improved D-gal-Induced Barrier Damage

D-gal induction has the potential to cause harm to the intestines, which includes compromising the intestinal barrier. Histological assessments utilizing H&E staining revealed that PM treatment markedly ameliorated the pathological damage induced by D-gal, including disarranged villi architecture, fractures, and apical detachment (Figure 7A). To enhance the understanding of intestinal barrier integrity, WB analysis was conducted to assess the levels of TJs proteins, particularly Occludin and ZO-1. In comparison to the Control group, a notable reduction in the levels of Occludin and ZO-1 was observed after treatment with D-gal. Importantly, administration of PM at both medium and high doses notably augmented the levels of these proteins in the hippocampus (P < 0.01, Figure 7B and C). Additionally, D-gal treatment resulted in damage to the BBB, which resulted in tight junction dysfunction; however, this effect was mitigated by PM treatment (Figure 7D). Moreover, the levels of Occludin and ZO-1 proteins in the hippocampus experienced a significant reduction after D-gal treatment, while PM treatment led to a notable increase in their expression (P < 0.05 or P < 0.01, Figure 7E and F).

# PM Treatment Modulates NF- $\kappa$ B and cAMP Signaling Pathways in the Hippocampus of D-gal-Induced CI Mice

The findings from the WB analysis indicated that, when contrasted with the D-gal group, there was a significant reduction in the relative expression levels of p-NF- $\kappa$ B-p65 in the hippocampal tissue (P < 0.01, Figure 8A–C). In contrast, there was a notable elevation in the levels of p-PKA and p-CREB (P < 0.05 or P < 0.01, Figure 8A–C). Further evaluations through ELISA revealed that treatment with PM resulted in a significant enhancement of both cAMP and SCFA concentrations within the hippocampus in comparison to the D-gal group (P < 0.01, Figure 8D). Moreover, IF studies demonstrated that PM treatment significantly increased the levels of p-CREB expression in the CA1 and CA3



**Figure 7** PM regulates the integrity of the barrier. (**A**) H&E staining of mouse intestinal tissues (scale bar = 200  $\mu$ m), (**B** and **C**) WB analysis of Occludin and ZO-1 in the intestine, (**D**) Representative TEM images of the hippocampal region (scale bar = 200 nm), (**E** and **F**) WB analysis of Occludin and ZO-1 in the hippocampus. Red arrows indicate villus breakage, black arrows indicate apical detachment of villi, and Orange arrows indicate TJs. n = 3. <sup>##</sup>P < 0.01 vs Control group; \*P < 0.05, \*\*P < 0.01 vs D-gal group.

regions (P < 0.05 or P < 0.01, Figure 8E and F). These results suggest that PM is involved in inhibiting the NF- $\kappa$ B inflammatory signaling pathway within the hippocampus while also promoting the cAMP signaling pathway.

#### PM Treatment Modulates Gut Microbiota in D-gal-Induced CI Mice

The rarefaction curve for  $\alpha$ -diversity, which is derived from the count of operational taxonomic units (OTUs) across various samples, levels off on the right, suggesting that saturation has been achieved in the sequencing data (Figure 9A). The assessment of  $\alpha$ -diversity, utilizing the Shannon and Simpson indices, revealed that the PM group demonstrated a



**Figure 8** PM Regulates NF- $\kappa$ B and cAMP Signaling Pathways. (**A**–**C**) WB analysis of p-NF- $\kappa$ B-p65, p-PKA, and p-CREB in the hippocampus, n=3, (**D**) Expression of SCFA and cAMP in the mouse hippocampus, n=6, (**E** and **F**) Immunofluorescence analysis and quantification of p-CREB (S133) in the CA1 and CA3 regions (scale bar = 20  $\mu$ m), n = 3. ##P < 0.01 vs Control group; \*P < 0.05, \*\*P < 0.01 vs D-gal group.



Figure 9 Continued.



Figure 9 PM Alleviates D-gal-Induced Gut Microbiota Dysbiosis. (A) Diversity rarefaction curve, (B) Diversity indices based on OTU levels, (C)  $\beta$ -diversity of gut microbiota based on Principal Coordinate Analysis (PCoA) and unweighted UniFrac algorithm, (D) Pie chart of OTU numbers for each group, (E) Bar chart at the phylum level, (F) Comparison of relative abundance of different phylum among groups, (G) *Firmicutes/Bacteroidetes* ratio, (H) Bar chart at the families level, (I) Comparison of relative abundance of different families among groups, (J) Bar chart at the genus level, (K) Comparison of relative abundance of different genus among groups. (L) LDA value distribution histogram and LEfSe circular phylogenetic tree. n = 5. #P < 0.05, #P < 0.01 vs Control group; \*P < 0.05, \*\*P < 0.01 vs D-gal group.

reduction in species richness compared to the D-gal group (P > 0.05, Figure 9B). Results from Principal Coordinate Analysis (PCoA) clearly displayed distinct separations between the D-gal, Control, and PM groups, highlighting significant differences in the gut microbiota structure of the PM group (Figure 9C). Additionally, the Venn diagram depicts the quantity of shared and distinct OTUs among the groups, revealing that there are 497 OTUs common across all three groups (Figure 9D).

Further analysis of the species diversity at different taxonomic tiers revealed notable changes in the gut microbiome. At the phylum level, the introduction of PM resulted in a substantial differences in the relative abundance of *Bacteroidetes* and *Proteobacteria* compared to the group treated with D-gal (P < 0.05, Figure 9E and F). Additionally, a noticeable trend was observed indicating a rise in *Actinobacteria* (P > 0.05, Figure 9E and F). Conversely, the treatment with PM corresponded to a significant decline in the relative abundance of *Firmicutes* (P < 0.01, Figure 9E and F), along with a lower *Firmicutes*/*Bacteroidetes* ratio, which suggests a modification in the composition of the microbiota (P < 0.05, Figure 9G). At the family

taxonomic level, the relative abundance of *Desulfovibrionaceae* decreased with PM treatment when compared to the D-gal group (P > 0.05, Figure 9H and I). In contrast, a marked reduction in the relative abundance of *Enterobacteriaceae* was observed following PM treatment (P < 0.05, Figure 9H and I). When analyzing the data at the genus level, there was a trend observed with PM treatment that indicated a rise in the relative abundance of *Bacteroides* and *Coprococcus* in comparison to the D-gal group (P > 0.05, Figure 9J and K). Notably, PM led to significantly higher relative abundances of *Prevotella, Anaeroplasma*, and *Clostridium* (P < 0.05 or P < 0.01, Figure 9J and K). These results demonstrate the significant impact of particulate matter on the composition of gut microbiota at various taxonomic levels.

The results suggest that PM significantly influences dysbiosis and modifies the alterations in gut microbiota caused by D-gal. Biomarkers with an LDA score greater than 4 were identified by employing linear discriminant analysis effect size (LEFSE) (Figure 9L). For the PM group, the identified biomarkers comprised *Parabacteroides* and *Gemmiger*, whereas the D-gal group presented biomarkers like [*Ruminococcus*] and *Ruminococcus*. Additionally, the PM group showed the presence of other biomarkers including *Bifidobacterium* and *Desulfovibrio*.

#### Discussion

Both PT and MO notably enhance CI and show promise as treatment options; their synergistic pharmacological effects can work in harmony, resulting in improved therapeutic results.<sup>33</sup> The pairing of PT and MO amplifies the soothing and cognitive-boosting benefits of PT, while MO contributes its dampness-drying and phlegm-eliminating attributes to support PT's expectorant actions. Together, they more effectively balance heart and kidney functions, thus alleviating symptoms associated with CL<sup>46,52,55</sup> Regarding the interactions and synergistic effects of the bioactive components of PT and MO, studies have shown that after their combination, significant changes occur in the content and composition ratio of chemical components, laying a material foundation for the synergistic effects.<sup>56</sup> MO can effectively alleviate the gastrointestinal motility inhibition induced by PT, and the mechanism may be related to the changes in component content.<sup>55</sup> In regulating gastrointestinal smooth muscle, their combination can significantly enhance the contraction amplitude, reduce the frequency inhibition rate, and weaken the inhibition on intestinal muscles.<sup>57</sup> PT polysaccharides alleviate gut microbiota dysbiosis and neuroinflammation in AD models by increasing the abundance of SCFA-producing bacteria (eg, Bacteroides) and decreasing pro - inflammatory bacteria (eg, Proteobacteria).<sup>58</sup> PT scavenges free radicals and reduces the increased intestinal permeability induced by oxidative stress.<sup>59,60</sup> The ethanol extract of MO alleviates intestinal inflammation and barrier damage by inhibiting the PI3K/AKT/NF-kB pathway.<sup>49</sup> Moreover, magnolol, the main component of MO, can reverse gut microbiota imbalance (eg, increasing Ruminococcaceae and decreasing Firmicutes) and improve lipid metabolism disorders (eg, reducing serum LPS and regulating bile acid metabolism), thereby protecting the intestinal barrier.<sup>61–64</sup> PT and MO have different focuses on the gut - brain axis. Their combination may optimize the microbiota structure, target oxidative stress and inflammatory cascades simultaneously, comprehensively supporting the hypothesis of PM's multi-target treatment for CI. These interactions can explain the enhanced effects of PM.

The fundamental principle by which D - gal induces CI is to simulate the biochemical changes of CI via long - term or high - dose D - gal injection. During the metabolic process in animals, a substantial amount of reactive oxygen species (ROS) is generated. This leads to an elevation in oxidative stress levels, the occurrence of neuroinflammation, neuronal damage, and a decline in cognitive function, thereby mimicking the process of human cognitive decline.<sup>65–69</sup> D-gal induction may also lead to the activation of intestinal inflammation. Pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IFN- $\gamma$  can enter the central nervous system through the blood circulation.<sup>70</sup> These cytokines act on nerve cells and glial cells in the brain, triggering neuroinflammation, affecting the synthesis, release, and metabolism of neuro-transmitters, interfering with neural signal transduction, and subsequently leading to cognitive impairment.<sup>71,72</sup> After LPS enters the blood circulation, it can activate the immune system and inflammatory signaling pathways, further exacerbating the systemic inflammatory response and indirectly affecting brain function, thus forming an intestinal-immune-brain signaling pathway.<sup>73,74</sup>

The imbalance of the gut microbiota caused by D-gal induction may lead to an increase in the level of intestinal oxidative stress, a decrease in SOD activity, and a reduction in GSH content.<sup>75</sup> Oxidative products generated by oxidative stress can reach the brain through the blood circulation, damaging neurons and glial cells and affecting neural plasticity

and neurotransmitter function.<sup>76</sup> Meanwhile, the oxidative stress response in the brain can also affect the redox state of the intestine through neuroendocrine signal feedback, forming an interaction between the intestine, oxidative stress, and the brain.<sup>77</sup> The research indicated that mice treated with D-gal displayed deficits in learning and memory when subjected to behavioral assessments, whereas PM significantly alleviated these impairments. Furthermore, pathological staining indicated that PM effectively countered the reduction in neuron counts and neuronal damage associated with D-gal treatment. As a reaction to the oxidative stress and the systemic inflammatory response triggered by D-gal, PM significantly reduced the levels of pro-inflammatory mediators such as TNF- $\alpha$  and IL-1 $\beta$  in the serum and hippocampus of subjects treated with D-gal. Additionally, PM significantly increased SOD activity and elevated GSH levels within the hippocampus, thereby mitigating oxidative stress in these mice. Moreover, PM reduced the content of pro - inflammatory substances in the serum. Through these pharmacological investigations, we established that PM was more effective than PT or MO individually in enhancing cognitive functions, pathological outcomes, and molecular markers in D-gal-treated mice. This highlights that the combination of PT and MO produces a pharmacological synergy, leading to improved therapeutic results.

The appropriate organization and operation of the intestinal barrier are associated with various diseases affecting the central nervous system, including Alzheimer's disease and Parkinson's disease.<sup>78</sup> In the CI model, D - gal induces damage to the intestinal barrier. The underlying mechanism may involve the production of a large amount of ROS, which triggers oxidative stress. Ultimately, this leads to apoptosis of epithelial cells or damage to the structure of TJs, thus disrupting the integrity of the intestinal barrier.<sup>79,80</sup> Research has shown that the active ingredients of traditional Chinese medicine can regulate the gut microbiota, significantly reduce the LPS content in the serum of model mice, improve the apoptosis and disordered arrangement of intestinal epithelial cells, upregulate the mRNA expression levels of ZO - 1, Occludin, and Claudin - 1, and contribute to the restoration of TJs between cells. This, in turn, helps maintain the integrity and permeability of the intestinal epithelial barrier.<sup>79,81,82</sup> SCFAs are produced by specific gut bacteria during the fermentation of dietary fiber. They provide energy for intestinal epithelial cells, promote their growth and differentiation, and are crucial for maintaining the integrity of the intestinal barrier.<sup>83</sup> In our study, PM increased the abundance of SCFA - producing bacteria such as Actinobacteria, Bacteroides, Prevotella, Anaeroplasma, and Clostridium, thereby enhancing the intestinal barrier function and maintaining the stability of the gut - brain axis. LPS is the main component of the cell wall of Gram - negative bacteria. Our research indicates that PM can decrease the abundance of LPS producing bacteria. When the intestinal barrier is impaired, the LPS generated by these bacteria enters the bloodstream, activating the immune system and triggering a systemic inflammatory response.<sup>84</sup> Subsequently, inflammatory cytokines can reach the brain via the bloodstream, activating microglia and astrocytes in the brain. These activated cells release a large number of pro - inflammatory cytokines, such as TNF - α, IL - 1β, etc., thus leading to neuroinflammation.<sup>85,86</sup> Neuroinflammation can damage neurons, affect the synthesis and release of neurotransmitters, disrupt neural signal transduction, and ultimately lead to cognitive impairment.<sup>87</sup> PM reduces the abundance of LPS - producing bacteria such as Proteobacteria, Desulfovibrionaceae, and Enterobacteriaceae, thereby decreasing the release of LPS. This alleviates both systemic and neuroinflammation, and further improves cognitive function. This demonstrates the impact of gut microbiota and their products on the brain's inflammatory state within the gut - brain axis.

BBB is a pivotal protective structure that shields the brain from noxious substances and the integrity of the BBB is intricately linked to cognitive function.<sup>88</sup> Damage to the BBB induced by D-gal may stem from different factors, such as the release of inflammatory cytokines, changes in astrocyte activation, and a decline in the expression of tight junction proteins among endothelial cells. These changes can significantly improve the permeability of the BBB, allowing LPS and pathogen molecules from the bloodstream to enter brain tissue, potentially leading to neuroinflammation and neurodegeneration.<sup>89,90</sup> Once in the brain, LPS stimulates brain endothelial cells and activates the caspase - 4/11 - GSDMD signaling pathway. This leads to the inflammatory disruption of the BBB, making it easier for harmful substances to enter the brain, thus further exacerbating neuroinflammation and neuronal damage.<sup>90</sup> In addition, LPS activates immune cells in the brain, such as microglia, triggering an inflammatory response in the brain. It also binds to Toll - like receptor 4, activating the downstream NF -  $\kappa$ B signaling pathway, which generates inflammatory cytokines like TNF -  $\alpha$ , IL - 1 $\beta$ , and IL - 6, thus contributing to the development of neurodegenerative diseases.<sup>91,92</sup> Although there are certain differences in tissue structure and physiological functions between the BBB and the intestinal barrier, they share

similar biological requirements in maintaining the stability of the body's internal environment and resisting the invasion of external harmful substances. Moreover, both rely on TJs to ensure the integrity and selective permeability of the barriers.<sup>93</sup> In this research, PM mitigated the pathological injury to the intestine in D-gal mice and restored the reduction in the levels of Occludin and ZO-1 in both the intestine and hippocampus induced by D-gal, thus safeguarding both the intestinal barrier and the BBB. This suggests that PM may have the potential to widely protect the body's barrier functions. On one hand, PM strengthens the BBB, reduces the entry of harmful substances into the CNS, decreases neuroinflammation and nerve damage, and improves cognitive function. On the other hand, it protects the intestinal barrier, prevents the translocation of LPS and pathogens, and maintains the homeostasis of the intestinal environment. Thus, it influences the functions and health of the brain through the gut - brain axis.

SCFAs are metabolites generated by the gut microbiota during the fermentation of dietary fiber. They can reach the brain via the bloodstream and are closely linked to the onset and progression of cognitive function and neurodegenerative diseases through the gut - brain axis.<sup>94</sup> In neurodegenerative diseases like AD and PD, a reduction in the quantity of SCFA - producing bacteria and a decrease in the concentration of SCFAs in feces have been observed.<sup>95</sup> SCFAs exert their physiological functions via GPCRs, especially GPR41 and GPR43. Activation of these receptors can trigger the activation of multiple intracellular signaling pathways, including that of adenylate cyclase, thus increasing the intracellular cAMP level and activating the cAMP signaling pathway.<sup>96</sup> SCFAs can impact the synthesis and metabolism of neurotransmitters, such as gamma - aminobutyric acid and serotonin, which are closely associated with cognitive function.<sup>97</sup> SCFAs also have immunomodulatory properties. They inhibit the production of inflammatory factors and promote the secretion of anti - inflammatory cytokines, thereby maintaining the balance of the immune system.<sup>98</sup> PM boosts the number of SCFA - producing bacteria, influences and regulates the brain's immune response through the gut - brain axis, mitigates the occurrence and development of neuroinflammation, shields neurons from inflammatory damage, and ultimately enhances cognitive function.

The transcription factor NF- $\kappa$ B is essential for cellular immune responses, inflammation, cell growth, and programmed cell death.<sup>99</sup> Activation of the NF- $\kappa$ B signaling pathway leads to the release of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, all of which play significant roles in neuroinflammation and CI. Inhibiting this NF- $\kappa$ B signaling pathway may reduce microglial activation and decrease neuroinflammation, which can subsequently improve cognitive function.<sup>100</sup> Moreover, the impairment of the blood-brain barrier has been associated with the triggering of the NF- $\kappa$ B signaling pathway, which may exacerbate cognitive impairment.<sup>100</sup> This research indicates that PM substantially lowered the levels of p-NF- $\kappa$ B-p65 in the hippocampal region of mice, alongside a decrease in pro-inflammatory cytokines in both the hippocampus and serum. These findings suggest that PM could mitigate CI induced by D-gal by inhibiting the NF- $\kappa$ B signaling pathway.

The cAMP signaling pathway plays an essential role as an intracellular mechanism that participates in numerous physiological functions, such as memory and learning, immune response, motor regulation, and metabolic processes.<sup>101</sup> CREB, an important transcription factor located in the nucleus, is predominantly found in the brain, especially in areas related to memory and learning, including the cortex and hippocampus. Changes in the expression levels of CREB might be associated with cognitive impairments.<sup>102</sup> For example, in AD, disruptions in the cAMP signaling pathway may result in lower phosphorylation levels of CREB, adversely affecting neuronal health and impairing learning and memory capacities.<sup>103</sup> cAMP plays a crucial role in supporting neuronal survival and regeneration via PKA, which contributes to the enhancement of cognitive abilities.<sup>104</sup> Neuroinflammation represents a significant pathological characteristic of AD, and influencing the cAMP signaling pathway might assist in regulating inflammation linked to this condition.<sup>105</sup> In our research, the administration of PM notably enhanced the levels of p-PKA and p-CREB in the hippocampal region of mice treated with D-gal, while also causing a significant increase in cAMP concentrations in this area. These findings suggest that PM stimulates the cAMP signaling pathway within the hippocampus of D-gal-treated mice and may mitigate cognitive deficits via this process.

Individuals with CI frequently encounter dysbiosis within their gut microbiota.<sup>106</sup> Numerous probiotic preparations can modulate the structure of the gut microbiota, increasing the relative abundance of beneficial bacteria such as Bifidobacterium and Lactobacillus. Moreover, they can alleviate intestinal inflammation and influence neurotransmitter metabolism.<sup>107,108</sup> The ratio of *Firmicutes* to *Bacteroidetes* serves as a widely utilized measure for evaluating the balance

of gut microbiota.<sup>109</sup> In healthy individuals, the gut microbiome predominantly consists of *Firmicutes* and *Bacteroidetes*, which together account for a considerable fraction of gut bacteria. The relative presence of these two phyla can indicate the condition of the gut microbiota, and any imbalance in this ratio might be associated with a range of diseases.<sup>110</sup> A heightened Firmicutes/Bacteroidetes ratio is linked to inflammatory responses and can impact both the gut and additional organs.<sup>111</sup> Our study found a notable decrease in the Firmicutes/Bacteroidetes ratio in mice that underwent D-gal treatment, in comparison to those in the Control group. This alteration was linked to heightened levels of inflammatory markers in both serum and the hippocampus and the administration of PM significantly mitigated this effect. Additionally, D-gal treatment resulted in lower levels of SCFAs within the hippocampus and an elevation of LPS concentrations in the serum. These effects may be linked to D-gal's disruption of gut microbiota balance, resulting in barrier impairment and heightened permeability of the BBB, which permits the LPS from specific gut bacteria to penetrate the intestinal lining and enter the bloodstream, thereby exacerbating BBB compromise and enter the brain to exacerbate the inflammatory response. This microbial imbalance reduces the population of beneficial bacteria that synthesize SCFAs leading to diminished SCFAs levels available in the hippocampus via circulation and consequently inhibiting the cAMP signaling pathway. In conclusion, PM has similar effects to probiotic preparations. By restoring the gut microbiota, inhibiting the NF - kB pathway, and activating the cAMP signaling pathway, PM alleviates cognitive impairment CI. This study has limitations. Mechanistic experiments did not directly compare PM with individual PT or MO, precluding clear distinction between synergistic and additive effects. The long - term impacts of PM are unassessed, and 16S rRNA sequencing limitations may bias gut microbiota and CI relationship analysis. Future research should extend the observation, and use multi-omics and more techniques to analyze PM's mechanisms. To validate the synergistic effects and explore PM's therapeutic potential in humans, experiments are needed to clarify the contributions of PT and MO, along with cellular assays and small-scale clinical trials.

### Conclusion

In this research, we showed that PM outperforms both PT and MO individually in enhancing CI. PM notably reduced the deterioration of learning and memory, neuronal injury, inflammation, and oxidative stress caused by D-gal. Our results suggest that PM mitigates CI by stimulating the cAMP-PKA and NF-κB signaling cascades, reinstating the integrity of the BBB and gastrointestinal barrier, as well as adjusting gut microbiota.

#### **Abbreviations**

AD, Alzheimer's disease; BBB, blood-brain barrier; CI, Cognitive impairment; cAMP, cyclic Adenosine Monophosphate; D-gal, D-galactose; ELISA, Enzyme-Linked Immunosorbent Assay; GPCRs, G-protein-coupled receptors; GSH, glutathione; H&E, Hematoxylin and Eosin; IF, Immunofluorescence; IFN- $\gamma$ , Interferon- $\gamma$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6, interleukin-6; LEFSE, Linear discriminant analysis effect size; LPS, Lipopolysaccharide; MWM, Morris Water Maze; NF- $\kappa$ B, Nuclear factor kappa-B; ANOVA, one-way analysis of variance; OTU, operational taxonomic unit; PAT, Passive avoidance test; CREB, p-cyclic Adenosine Monophosphate Response Element Binding; PCoA, principal co-ordinates analysis; PKA, Protein Kinase A; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; SOD, superoxide dismutase; TJs, tight junctions; TEM, Transmission Electron Microscopy; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; UPLC-MS/MS, ultra-high performance liquid chromatography-tandem triple quadrupole mass Spectrometry; WB, Western blotting; ZO-1, Zonula occludens protein 1; A $\beta$ ,  $\beta$ -protein.

#### **Data Sharing Statement**

The datasets PRJNA1191803 for this study can be found in the National Center for Biotechnology Information (NCBI). https://www.ncbi.nlm.nih.gov/sra/PRJNA1191803.

## Funding

This research was funded by the National Natural Science Foundation of China (82003965), Chengdu University of Traditional Chinese Medicine - Xinglin Scholar Program (QJRC2022033), Sichuan Provincial Administration of Traditional Chinese Medicine Project (2024MS167), The Seventh Batch of National Academic Experience Inheritance

Project for Old Chinese Medicine Experts by the National Administration of Traditional Chinese Medicine {Guo Zhong Yi Yao Ren Jiao Han (2022) No. 76}, National-level project of the College Student Innovation and Entrepreneurship Training Program (202310633028) and Provincial-level project of the College Student Innovation and Entrepreneurship Training Program (S202410633056).

#### Disclosure

The authors report no conflicts of interest in this work.

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