



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Polygonum multiflorum-derived tetrahydroxy stilbene glucoside alleviates APP/PS1 mice by regulating gut microbiota and feces metabolomics

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ABSTRACT: Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive cognitive decline. Tetrahydroxy stilbene glucoside (TSG) has been demonstrated to improve learning and memory in aged mice; however, its underlying mechanisms remain incompletely understood. This study aimed to elucidate the effects of TSG on cognitive impairment in APP/PS1 mice through analysis of gut microbiota and associated metabolites. Behavioral tests, immunohistochemistry, and 16S rDNA sequencing revealed that TSG treatment improved cognitive function and alleviated neuroinflammation. Furthermore, TSG restored gut microbiota homeostasis and normalized aberrant metabolite profiles, accompanied by elevated levels of short-chain fatty acids (SCFAs). Correlation analysis indicated associations between alterations in gut microbiota, metabolites, and SCFAs. Notably, TSG promoted the production and content of SCFAs, especially acetic acid, propionic acid, and hexanoic acid. These findings suggest that TSG mitigates AD-related pathology possibly via modulation of specific gut microbial communities and their metabolic outputs, providing a basis for further therapeutic exploration.

Keywords: Tetrahydroxy stilbene glycoside; Alzheimer's disease; Gut microbiota; Neuroinflammation; Short-chain fatty acids

1. Introduction

As the leading cause of dementia, Alzheimer's disease (AD) is a progressive neurodegenerative condition whose hallmark is debilitating cognitive dysfunction and progressive memory impairment [1-3]. The amyloid cascade hypothesis, which has dominated AD pathology for decades, suggests that amyloid- β (A β) accumulation is the primary initiating factor [4-6]. Yet, the repeated failure of over 30 Phase III clinical trials targeting A β to mitigate cognitive decline challenges this paradigm and underscores the complexity of the

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Received 13 November 2025

Received in revised form 2 December 2025

Accepted 13 January 2026

disease. This unmet therapeutic need highlights the imperative to explore novel biological mechanisms and develop safer, more effective treatment strategies.

Mounting research underscores the involvement of the gut microbiota in a range of neurological conditions, including multiple sclerosis, Parkinson's disease, and AD [7-9]. Disruption of the microbiota-gut-brain axis—whereby changes in microbial composition and metabolism affect central nervous system (CNS) function—can contribute to disease development [10]. The gut flora directly modulates brain activity and neuronal function, with microbial diversity being critical for preventing behavioral abnormalities and ensuring proper microglial activation. In AD, although the etiology is multifactorial, gut microbes, notably key phyla like *Proteobacteria*, *Firmicutes*, and *Bacteroidota* [11] are believed to exert effects through their metabolic output. These shifts in the microbial ecosystem can initiate or accelerate pathology [12]. Presently, however, studies examining bacteria in AD have mainly described differences in microbial taxa across individuals.

As key microbial metabolites, short-chain fatty acids (SCFAs) are implicated in CNS pathologies through their regulation of neuroinflammation, neuronal apoptosis, and oxidative stress [13-15]. SCFAs help preserve intestinal low permeability and blood-brain barrier (BBB) integrity while also supporting microglial and astrocytic functions [16, 17]. Preclinical evidence links SCFAs depletion to AD progression, whereas SCFAs administration ameliorates oxidative injury, cognitive impairment, and A β accumulation, core pathological hallmarks of AD, in murine models [18]. Therefore, modulating gut microbiota and their metabolic output, especially SCFAs, is emerging as a potential therapeutic avenue for neuroinflammatory and neurodegenerative conditions [19]. Strategies to improve microbial ecology, suppress inflammation, and regulate the gut-brain axis hold significant promise for delaying or preventing AD.

Plant glycoside extracts serve as natural precursors for intestinal SCFAs and possess diverse pharmacological properties, including antioxidant, immunomodulatory, and anti-aging activities [20, 21]. Compared to synthetic drugs, these phytochemicals offer superior safety profiles with minimal side effects, highlighting their potential in preventing and treating AD. Tetrahydroxy stilbene glucoside (TSG), a primary active component isolated from the traditional Chinese medicine (TCM) *Polygonum multiflorum*, is noted for its memory-enhancing and anti-inflammatory effects, particularly against chronic inflammatory conditions [22]. However, the mechanisms through which TSG exerts its therapeutic actions in inflammatory diseases, including AD, are not fully elucidated. Therefore, this study aims to investigate the essential role of TSG in AD, providing new insights into its underlying mechanisms.

AD related cognitive and behavioral deficits are implicated with intestinal flora. While TSG is known to protect the CNS by reducing A β deposition and enhancing autophagic clearance, its influence on the gut microbiota in AD remains unexplored. Although TSG has been proven to have neuroprotective effects, whether it affects AD by regulating the gut microbiota-SCFAs-brain axis has not been reported. Therefore, the current study investigated the anti-inflammatory effects of TSG in male APP/PS1 mice to elucidate its mechanism of action, with a focus on its association with gut microbiota modulation. Notably, TSG promoted the production and content of SCFAs, especially acetic acid, propionic acid, and hexanoic acid. These findings

offer new insights into the role of gut microbes in AD pathogenesis, advance the understanding of TSG's neuroprotective effects, and lay a foundation for developing TSG related therapeutics.

2. Materials and methods

2.1. Materials and reagents

The chemical structure of TSG is shown in Figure 1. TSG (purity > 98%) was obtained from Chengdu Pufei De Biotech Co., Ltd. (Sichuan, China). HPLC-grade solvents, including acetonitrile, methanol, and formic acid, were purchased from Fisher Scientific (Fairlawn, NJ, USA). Deionized water for LC-MS analysis was prepared using a Milli-Q water purification system (Milford, MA, USA). All other reagents used in this study were of analytical grade.

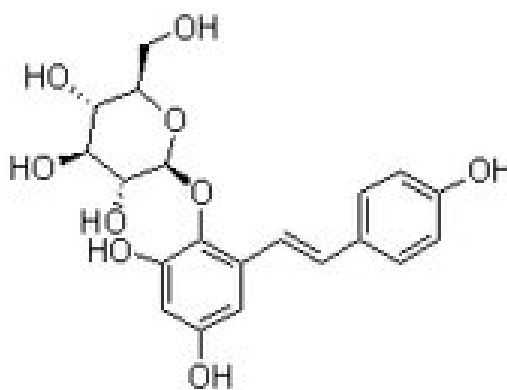


Fig. 1. Chemical structure of TSG.

2.2 Animals and experiment design

Five-month-old APPswe/PSEN1dE9 (APP/PS1) double-transgenic mice and their wild-type (WT) littermates were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). These mice, generated on a C57BL/6 background, harbor human APPswe and PSEN1-dE9 mutations. After a one-week acclimation period under standard housing conditions with ad libitum access to food and water, the mice were randomly assigned to five groups: a WT control group, an APP/PS1 model group, and three APP/PS1 groups treated with TSG at doses of 60, 120, and 180 mg/kg. TSG was administered orally every other day for eight weeks, while the WT and model control groups received an equivalent volume of vehicle. All animals were euthanized at the end of the two-month treatment period.

By conducting simulations based on prior data and starting from the widely accepted equilibrium point within the field, a reasonable foundation is provided for detecting moderate effect sizes. Through strict matching (such as age, weight, and litter allocation), the variability is minimized to the greatest extent, and ultimately the number of samples in each group is determined.

All the experiments were approved by the Animal Care Committee of Capital Medical University (Certification No.: AEEI-2025-14). The authors made every effort to minimize the total number of all the mice.

2.3 Sample collection and preparation

Mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with physiological saline. Following perfusion, the brains were rapidly dissected and removed. The brain tissues were then either snap-frozen for subsequent biochemical and molecular analysis or fixed in paraformaldehyde for morphological studies.

Fresh fecal samples were collected on final day and immediately flash-frozen in liquid nitrogen. After 12 hours, all samples were transferred to a -80 °C freezer for long-term storage. For metabolomic profiling, frozen samples were thawed on ice and homogenized in a three-fold volume of ice-cold methanol. The homogenates were then sonicated for 10 minutes and centrifuged (15,000×g, 15 min, 4 °C), with the resulting supernatant collected for analysis. For 16S rDNA sequencing, samples were aseptically collected under a nitrogen atmosphere to maintain anaerobiosis and stored at -80 °C until DNA extraction.

2.4 RNA sequencing

Total RNA was prepared from frozen tissue and used to generate a sequencing library by the Illumina MiSeq desktop sequencer and the Illumina HiSeq2500 (Beijing Cnkingbio Biotechnology Corporation, China). Gene analysis was performed by using ingenuity pathway analysis (IPA) methods (Tri-I Biotech, Shanghai, China).

2.5 Bone marrow-derived macrophages preparation

Bone marrow-derived macrophages (BMDMs) were prepared from L929 fibroblast-conditioned medium, which secretes macrophage colony-stimulating factor (M-CSF) essential for monocyte differentiation, following an established protocol [23]. The resulting BMDMs were then stimulated with LPS (100 µg/mL) or LPS plus IFN γ (20 ng/mL) for 6 hours, with or without TSG (250 ng/mL).

2.6 Primary microglia culture

Primary microglia were obtained from the prefrontal cortex and hippocampus of neonatal (1–2 day-old) mice. Brain tissues were disinfected, dissected, and digested in 0.25% trypsin-EDTA with DNase (1 mg/mL) at 37 °C for 10 minutes. After centrifugation and washing, cells were suspended in complete DMEM/F12 medium (10% FBS, 1% penicillin-streptomycin) and cultured in 75 cm² flasks. Medium was changed every three days. Microglia were isolated after 10 days by shaking at 150 r/min for 5 hours, and purity was verified by Iba1 immunostaining.

2.7 ELISA

TNF- α and IL-6 of BMDMs cultural supernatant were measured by ELISA kit (Becton, Dickinson and Company, USA) according to manufacturer's guidance. Briefly, standards and diluted supernatant samples were loaded onto a pre-coated 96-well plate. Following sequential incubations and wash steps to capture target

cytokines and bind a detection antibody, a colorimetric signal was generated using a horseradish peroxidase (HRP) substrate. The enzymatic reaction was terminated, and the absorbance was measured at 450 nm.

2.8 Immunofluorescence

Cells were immunolabeled with an anti-Iba1 primary antibody (1:1000, WAKO) following Fc receptor blockade with anti-CD16/32 (1:1000, Abcam). A fluorescent secondary antibody (1:500, KPL) was applied for detection. Nuclei were visualized with DAPI, and images were captured by laser scanning confocal microscopy fluorescence microscopy (Leica TCS SP2, Germany).

2.9 Hematoxylin & Eosin

Following fixation in 10% buffered formalin, brain and colon tissue samples were processed through a series of dehydration and clearing steps before being embedded in paraffin blocks. Sections of 4 μ m thickness were cut from these blocks and stained with hematoxylin and eosin (H&E) according to established protocols. Bright-field images of the stained sections were acquired with a light microscope, and subsequent densitometric analysis was performed using ImageJ software.

2.10 Immunohistochemistry

Following transcardial perfusion with saline and 4% PFA, mouse brains were post-fixed, sectioned, and permeabilized with PBST. Sections from the hippocampus, cortex, and striatum were immunostained using an anti-A β primary antibody (1:500, 4 °C, overnight) and a corresponding secondary antibody (1:500, 2 h, RT). The sections were then dehydrated, cleared, and cover slipped for microscopic analysis by using digital medical image analysis system Image-Pro Plus software (Media Cybernetics, Silver Spring, MA, USA).

2.11 Behavioral test

2.11.1 Morris water maze (MWM)

Spatial learning and memory were evaluated using the Morris water maze. Mice underwent 5 consecutive days of hidden-platform training, consisting of 4 trials per day with a maximum duration of 60 seconds per trial and an inter-trial interval of 20 minutes. The hidden platform remained fixed in the center of the southwest quadrant. For each trial, the entry point into the pool was randomly selected from the four cardinal starting locations (north, east, south, and west), ensuring that all four points were used once per day without repetition. Twenty-four hours after the final training session, a 60-second probe test was conducted with the platform removed. In the acquisition phase, mice were trained to find a hidden platform within 60 seconds from varying start quadrants. Mice that failed were guided to the platform and remained for 20 seconds. Upon completion of training, a probe test was performed with the platform removed, allowing 60 seconds of free exploration from the quadrant opposite the previous platform location.

2.11.2 Novel object recognition (NOR)

Working memory was assessed using the novel object recognition (NOR) test in an open black arena. Mice were first habituated to the empty arena for 10 minutes. After a 20-minute rest, they underwent a 6-minute training session with two identical objects. Twenty-four hours later, one object was replaced with a novel one for a 6-minute test session. A discrimination index was calculated from both phases to quantify object recognition.

2.12 16S rDNA

2.12.1. Extraction of genome DNA

Genomic DNA was extracted with the CTAB/SDS method, checked on 1% agarose gels, and diluted to 1 ng/ μ L. Target regions (16S V3-V4: 341F/806R; 18S V9: 1380F/1510R; ITS1: ITS1F/ITS2R) were amplified in 30 μ L reactions containing Phusion® High-Fidelity Master Mix, 0.2 μ mol/L primers, and ~10 ng DNA. The PCR protocol was: 98 °C for 1 min; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 60 s; and a final 72 °C for 5 min.

2.12.2. PCR performance

PCR amplicons were verified by 2% agarose gel electrophoresis. Those with a prominent band at 400-450 bp were selected, pooled in equal densities, and purified (AxyPrep DNA Gel Extraction Kit, UC, USA). Libraries were prepared (NEB Next® Ultra™ DNA Library Prep Kit, MA, USA) and sequenced on an Illumina MiSeq/HiSeq 2500 platform, producing 250/300 bp paired-end reads.

2.12.3. Data analysis

Raw paired-end sequences were merged and assigned to samples via barcode demultiplexing in FLASH. The UPARSE pipeline was employed for OTU clustering at 97% sequence similarity. Alpha diversity indices (Chao1, Observed Species, Shannon) were calculated from rarefied OTU tables using custom Perl scripts. Beta diversity was assessed through principal coordinates analysis (PCoA) of weighted and unweighted UniFrac distances within QIIME. Taxonomic composition was visualized with Krona charts. Differential abundance analysis across groups was performed using STAMP, while community-level differences were statistically tested with ANOSIM and ADONIS based on Bray-Curtis dissimilarity matrices.

2.13 Fecal SCFAs analysis

Fecal SCFAs were derivatized and analyzed by GC-MS. Separation used an Agilent DB-FFAP column (30 m $\times$ 250 μ m $\times$ 0.25 μ m) with helium (1.0 mL/min) and a temperature program from 90 °C to 240 °C. Detection was performed on an Agilent 5977B MSD, UC, USA. Quality control samples were interspersed to monitor analytical performance.

2.14 Statistical analysis

All data are expressed as mean $\pm$ SEM. Differences between groups were analyzed by Student's t-test or one-way ANOVA using GraphPad Prism 8.0. A P -value < 0.05 was regarded as statistically significant.

Biochemical parameters, behavior test, and protein expression data from mouse and cell samples were analyzed by GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA). All data are expressed as mean $\pm$ SEM. Differences between three or five groups were analyzed by one-way ANOVA to determine overall significance. When ANOVA indicated significant differences, Tukey's post hoc test was applied for multiple comparisons to identify specific group differences. For protein expression data, fold changes relative to the control group were calculated and analyzed similarly. Additionally, Pearson's correlation coefficient was used to assess relationships between biochemical and protein expression data. A P -value < 0.05 was regarded as statistically significant for all analyses.

3. Results

3.1. TSG ameliorated cognitive deficits in AD mice without toxicity

Firstly, within a period of two months, the changes of weight in mice were not significant (Fig. 2A and 2B). Based on our previous research experience, we have chosen the TSG doses as 60 mg/kg, 120 mg/kg, and 180 mg/kg [24-26]. In order to assess potential therapeutic function of TSG, mice were subjected to an MWM test to evaluate cognitive function and learning memory. Spatial learning deficits in APP/PS1 mice were confirmed by their significantly longer escape latencies and inefficient swimming trajectories compared to WT controls. These deficits were significantly ameliorated by TSG intervention. A progressive reduction in escape latency was observed across all groups over the 5-day acquisition phase; however, the TSG-treated AD group consistently demonstrated superior performance, achieving significantly shorter latencies relative to the untreated AD model (Fig. 2C and 2D). Schematic diagram of NOR test is shown in Fig. 1E. Compared to WT group, NOI in model group was significantly decreased, indicating that impaired memory ability and shorter time for exploring new objects (Fig. 2F and 2G). In contrast, the NOI and time exploring objects in TSG-treated groups were increased compared to model group.

Histopathological analysis revealed distinct morphological differences in brain tissues. Normal mice exhibited intact hippocampal neurons with dense and orderly arrangement. In contrast, AD model mice demonstrated substantial neuronal disorganization and loss, marked by widespread neuronal shrinkage. After TSG intervention, the mice showed reduced histopathological damage, normal appearance and increased number of neurons, and the effects of TSG were more obvious (Fig. 2H). Next, we prioritized evaluating A β deposition in mouse brain. The A β deposition was visualized by the antibody (Fig. 2I). Comparative histopathological examination indicated a substantially greater A β plaque burden in the hippocampi of APP/PS1 mice versus WT controls. A pronounced reduction in amyloid deposition was observed subsequent to TSG treatment. This clearance of A β aggregates is concomitant with the mitigation of cognitive deficits, supporting the therapeutic efficacy of TSG in this AD model.

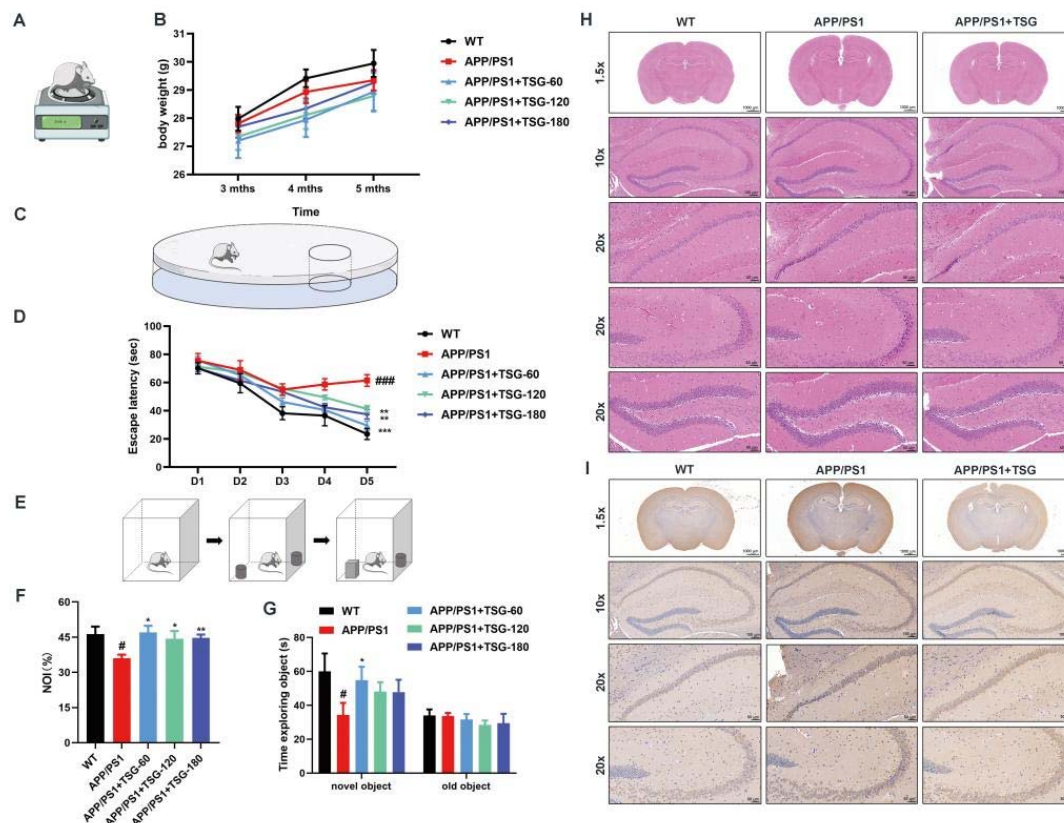


Fig. 2. TSG improved cognitive deficits without causing toxicity in AD mice. (A) Schematic diagram of body weight. (B) Body weight during 8 weeks. (C) Schematic diagram of MWM test. (D) Line chart of the changes in escape latency during training period. (E) Schematic diagram of NOR test. (F) Representative diagram of NOI. (G) Histogram of the percentage of exploring time of mice in each group. (H) Brain H&E staining to observe the effect of TSG on inflammation infiltration. (I) A β to observe the effect of TSG on decreasing the number and area of plaque. Data are presented as mean $\pm$ SEM. $^{\#}P < 0.05$, $^{###}P < 0.001$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs APP/PS1 model group. MWM, morris water maze; NOR, novel object recognition; NOI, novel recognition index.

3.2 TSG alleviated inflammatory response and neuronal morphology

We compared the differences in inflammatory-related factors among various groups in the hippocampus of mice by RNA-seq (Fig. 3A), and verified these findings in an *in vitro* experiment. By analyzing the supernatant of BMDMs, it was found that TSG could effectively reduce the expression of TNF- α and IL-6 (Fig. 3B and 3C). Considering the influence of pro-inflammatory cytokine secretion and antioxidant enzymes, the concentration of these factors including superoxide dismutase (SOD), adenosine triphosphate (ATP), glutamate, malondialdehyde (MDA), reactive oxygen species (ROS), lactic dehydrogenase (LDH) and intracellular iron were measured by kits (Fig. 3D to 3J). After LPS or LPS+IFN γ stimulated BMDMs for 6 h, LPS or LPS+IFN γ inhibited the content of SOD, ATP, Fe $^{2+}$ and glutamate, whereas promoted the release of ROS, MDA, and LDH. Conversely, TSG (250 ng/mL) treatment effectively attenuated the elevated levels of these pro-inflammatory cytokines and oxidative enzymes, demonstrating significant anti-inflammatory and antioxidant activities. Collectively, these findings indicate that TSG mitigates neuroinflammation and oxidative stress, thereby conferring neuroprotective effects.

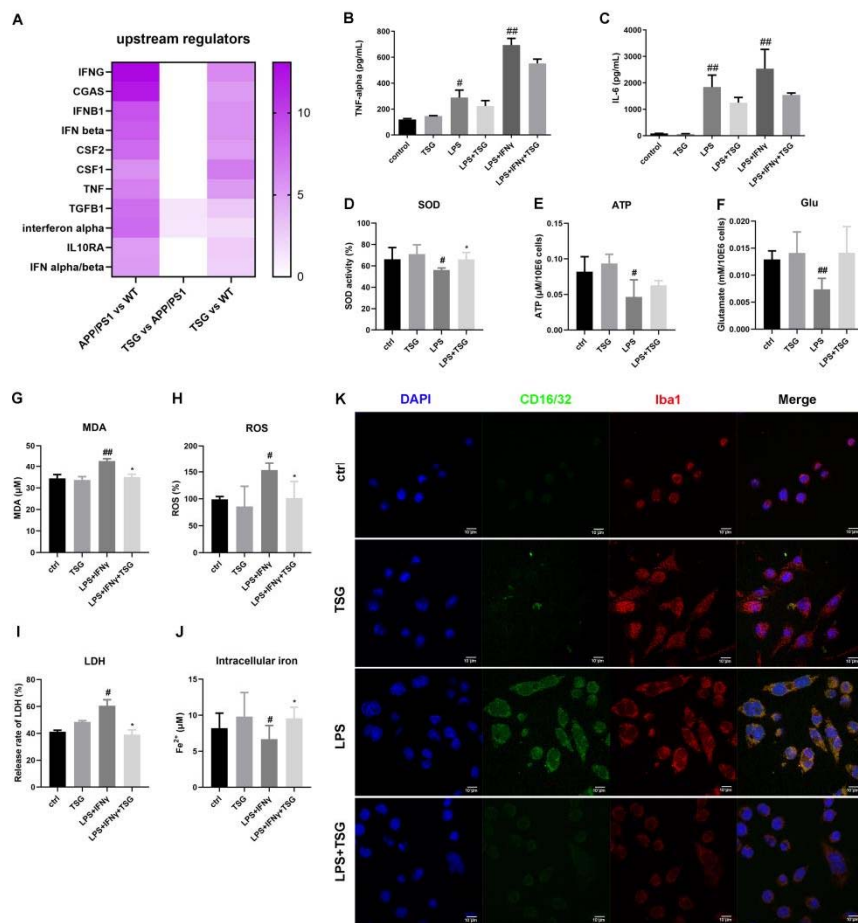


Fig. 3. TSG alleviated inflammatory enzymes and protected the neuronal morphology in cell. (A) Mouse brain transcriptome data related to inflammatory factors. (B) TNF- α and (C) IL-6 of BMDMs supernatant. (D) SOD, (E) ATP, (F) Glutamate, (G) MDA, (H) ROS, (I) LDH, and (J) intracellular iron of BMDMs. (K) Detection of inflammation in primary microglia by immunofluorescence. CD16/32 (green), Iba1 (red), and DAPI (blue). Groups: ctrl, TSG, LPS, LPS+TSG. Scale bar: 10 μ m. Data are presented as mean $\pm$ SEM. $^{\#}P < 0.05$, $^{##}P < 0.01$ vs control group; $^{*}P < 0.05$ vs APP/PS1 model group.

Changes in the inflammatory response were further assessed in primary microglial cultures derived from the prefrontal cortex and hippocampus of neonatal mice. LPS induced the aggravation of inflammation in cells. However, TSG alleviated disorder of inflammation, suggesting that it hold a certain anti-inflammatory effect (Fig. 3K). Based on our findings, we propose that TSG exerts its neuroprotective action primarily by inhibiting microglial activation.

3.3 TSG altered alpha and beta diversity of gut microbiota in AD mice

To analyze the bacterial composition and assess the degree of microbial dysbiosis, we performed 16S rDNA gene sequencing [27]. Sequencing analysis of the 16S rDNA gene revealed that TSG significantly altered the gut microbiota composition, prompting further investigation into its association with AD pathogenesis. The sequencing depth has covered the species in sample, and the sequencing data are valid, as evidenced by the original sequencing information. The unique features of each group were presented in Venn diagram. Sequences between WT and APP/PS1 groups were divided into the same OTU as 703 while between APP/PS1 and TSG 120 mg/kg+APP/PS1 groups were 652 (Fig. 4A).

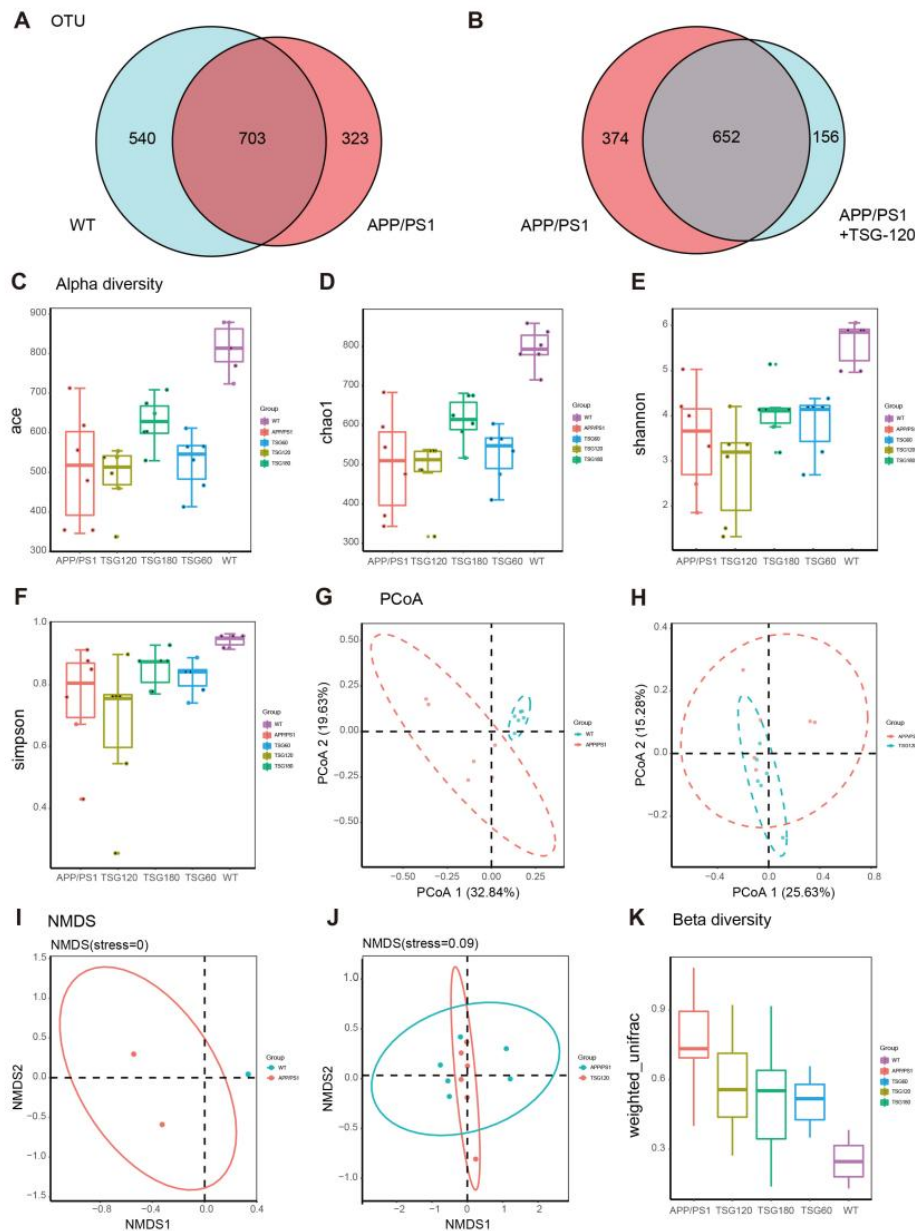


Fig. 4. TSG altered α - and β -diversity of gut bacteria in AD mice. (A, B) OTU Venn analysis between APP/PS1 vs WT and TSG 120 vs APP/PS1. Each circle represents a group. α -Diversity of different groups of gut bacteria at the level of OTU; the values represent species diversity. Variations in Ace (C), Chao (D), Shannon (E), and Simpson (F) diversity indices between different groups. PCoA (G, H) and NMDS (I, J) of microbiota in different groups. (K) β -diversity of different groups of gut bacteria at OTU level. Wilcoxon rank sum test; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; NMDS, non-metric multidimensional scaling.

Alpha and beta diversity were compared to assess variations in bacterial diversity among groups, measuring within-sample richness and between-sample community differences, respectively. A decrease in microbiota richness estimated by Ace, Chao, Shannon and Simpson indices were observed in model group. By group comparison, we found a clear distinction among various groups in Fig. 4B to 4F. Moreover, richness of bacterial communities in sample was estimated as presented by the rarefaction curve. The saturated shape of rarefaction curves indicated that bacterial richness of sample was completely sampled. Beta diversity was used to analyze the differences. Through weighted unifracBeta, there were significant differences between groups (Fig. 4K). The bacterial β -diversity has been examined utilizing PCoA (Fig. 4G and 4H) and NMDS. Applying NMDS analysis, the information about the species contained in the sample is reflected in the form

of points on a multidimensional space (Fig. 4I and 4J). Besides, the degree of variation between various samples is reflected by the distance between points, which could reflect the inter- and intra- group differences of sample.

3.4 TSG affected gut microflora classification and diversity in AD mice

Following taxonomic classification of OTUs against a specialized database, bar plots were constructed to depict the relative abundances at the phylum, class, order, family, genus, and species levels. The resultant structural distribution of the bacterial communities for the respective experimental groups is illustrated in Fig. 5A to 5F.

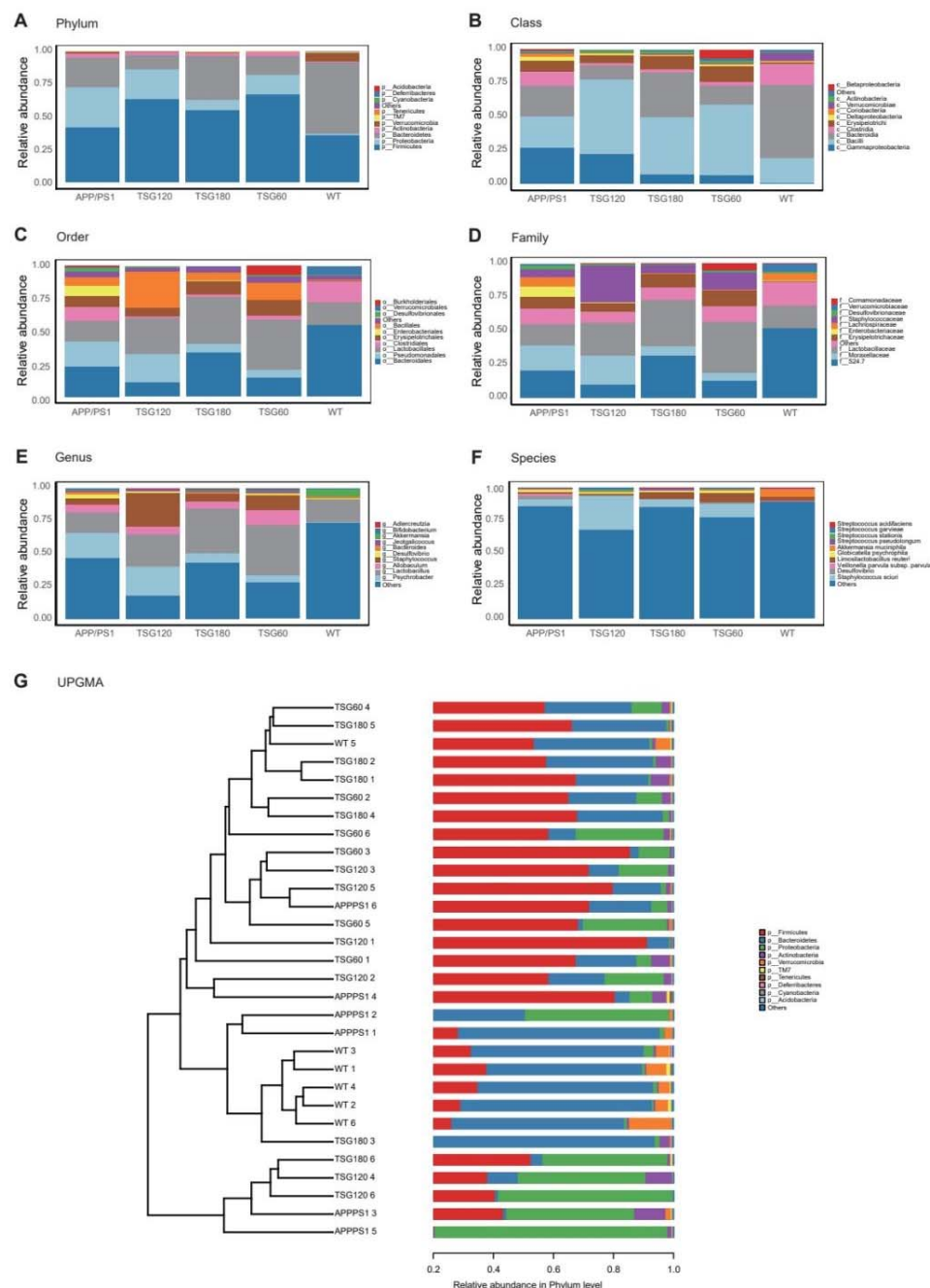


Fig. 5. TSG altered gut microbiota abundance in AD mice. The abundance of bacterial communities in AD mice at the (A) phylum, (B) class, (C) order, (D) family, (E) genus, and (F) species levels. (G) UPGMA clustering tree and community structure. TSG altered the abundance of gut microbiota in AD mice and the ratio of gut microbiota at different levels.

In these groups, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the predominant phyla. *Proteobacteria* in model group was significantly more abundant than in TSG and WT groups, whereas the abundances of *Bacteroidetes* were less abundant and considerably elevated in model groups (Fig. 5A). The class top 10 samples in different groups were shown in Fig. 5B. WT group has the highest *Verrucomicrobia* and *Bacteroidia*; different doses of TSG have the highest proportion of *Deltaproteobacteria* and *Bacilli*; APP/PS1 has the highest proportion of *Gammaproteobacteria*. At the order level, WT group has the highest *Bacteroidales*; different doses of TSG have the highest *Lactobacillales* compared to model group (Fig. 5C). At the family level, WT group has the highest *S24.7* and others; different doses of TSG have the highest *Lactobacillaceae* (Fig. 5D). At the genus level, different doses of TSG have lower *Psychrobacter* than model group (Fig. 5E). At the species level, WT group has the highest *muciniphila* than other groups (Fig. 5F).

To investigate inter-sample similarities, we performed cluster analysis. By UPGMA of weighted unifrac-dm tree barplot analysis, TSG groups have the highest proportion of *Firmicutes*; WT and TSG treatment group has the highest proportion of *Verrucomicrobia* and *Bacteroidetes*; APP/PS1 group has the highest proportion of *Proteobacteria* in Fig. 5G. Taken together, these results above were similar in both categories.

The taxa tree of the groups (top 10) was shown in Supplement Fig. 1, suggesting that WT has the highest proportion of *Verrucomicrobia*, which is really common; APP/PS1 has the highest proportion of *Proteobacteria*, which is also evidenced by the following data; TSG 120 mg/kg+APP/PS1 has the highest proportion of *Bacteroidetes*. All kinds of details of bacteria could refer to the figure.

3.5 TSG mediated the function prediction and correlation analysis in neuroinflammation

To analyze functional differences between sample groups, we used PICRUSt software to predict metagenomic functions based on 16S rRNA gene sequencing data. COG column diagram revealed the functional composition of the bacteria and COG predicted of barplot presented that the model group was obviously changed from other groups (Fig. 6A and 6C). It seems that the “membrane transport” changed the most in APP/PS1 model group. KEGG functional prediction analysis is an effective way to study metabolic changes of community samples in order to adapt to environmental changes. We chose the top 10 function prediction of KEGG in barplot and KEGG predicted of heatmap (Fig. 6B and 6D).

By COG predicted of heatmap analysis, the up and down regulated biological function changed a lot in APP/PS2 groups, especially “secondary metabolites biosynthesis, transport, and catabolism”, “cytoskeleton”, and “replication, recombination and repair” (Fig. 6B). But TSG treatment alerted this kind of situation as “cell motility” and “extracellular structures”. By KEGG predicted of heatmap analysis, the most upregulated ones as “membrane transport” and “metabolism”, and downregulated ones as “cell growth and death” and “translation” in model group (Fig. 6D).

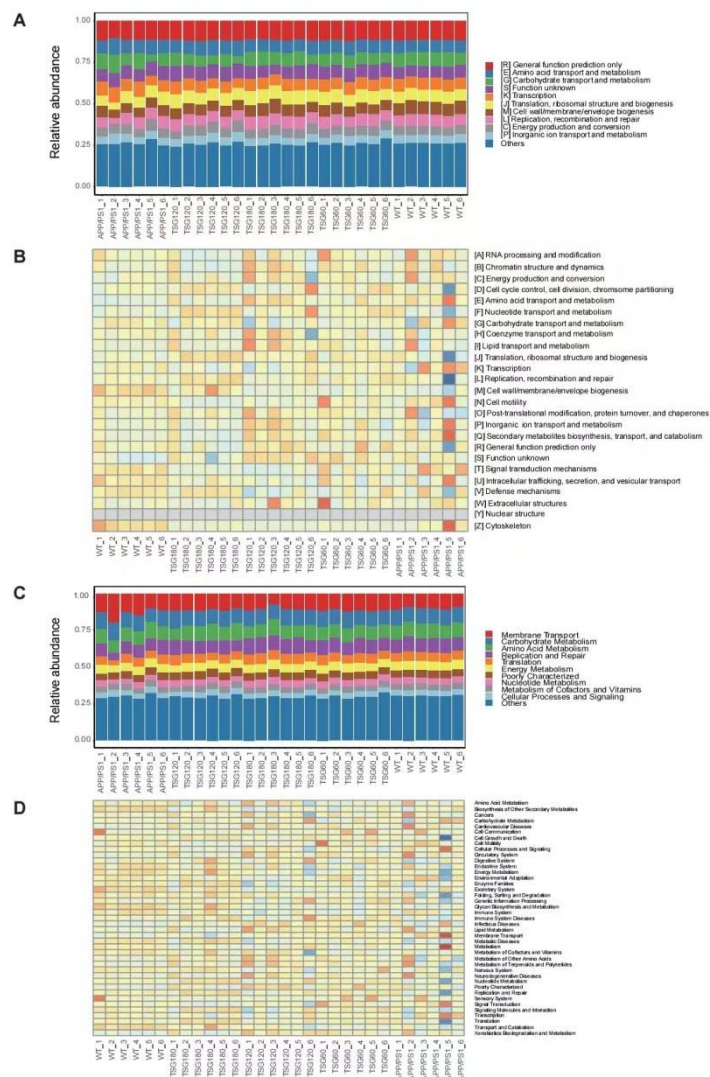


Fig. 6. TSG altered the function prediction and correlation by COG and KEGG. Function prediction including of (A) COG predicted of Barplot, (B) COG predicted of heatmap, (C) KEGG predicted of Barplot, and (D) KEGG predicted of heatmap between APP/PS1 vs WT and TSG 120 vs APP/PS1.

3.6 TSG regulated the composition and function of gut microflora

The Linear Discriminant Analysis Effect Size (LEfSe) method was performed to identify taxa with significant differential abundance across predefined groups, thereby pinpointing biomarkers that effectively distinguish sample categories. The relative results were shown as cladogram, the biomarkers, STAMP and t-test. To identify the differential gut microbiota between the groups, we used LEfSe methods to analyze the differences (Fig. 7A and 7B) and the biomarkers exhibited obvious difference.

By cladogram, *Proteobacteria*, *Firmicutes*, *Bacillales*, *Bacteroidales* and *Verrucomicrobia* were downregulated in WT group than APP/PS1 group, while *Bacilli*, *Actinomycetales*, and *Gammaproteobacteria* were upregulated in APP/PS1 group in Fig. 7C. Compared TSG 120 mg/kg+APP/PS1 group and APP/PS1 model group, *Clostridia*, *Lachnospiraceae* and *Veillonellaceae* changed the most in Fig. 7D. According to LDA score, we compared the LDA among the groups and found that it was roughly consistent with the above results. The detailed differences are shown in Fig. 7E and 7F. Finally, the most changed mean between WT and APP/PS1 group was *Akkermansi* ($P=0.0194$), followed with *Turicibacter* ($P=0.0047$) and *Oscillospira*

($P=0.0217$). Between TSG 120 mg/kg+APP/PS1 and APP/PS1 group, the most changed mean was *Staphylococcus* ($P=0.0323$) by t-test in Fig. 7G and 7H.

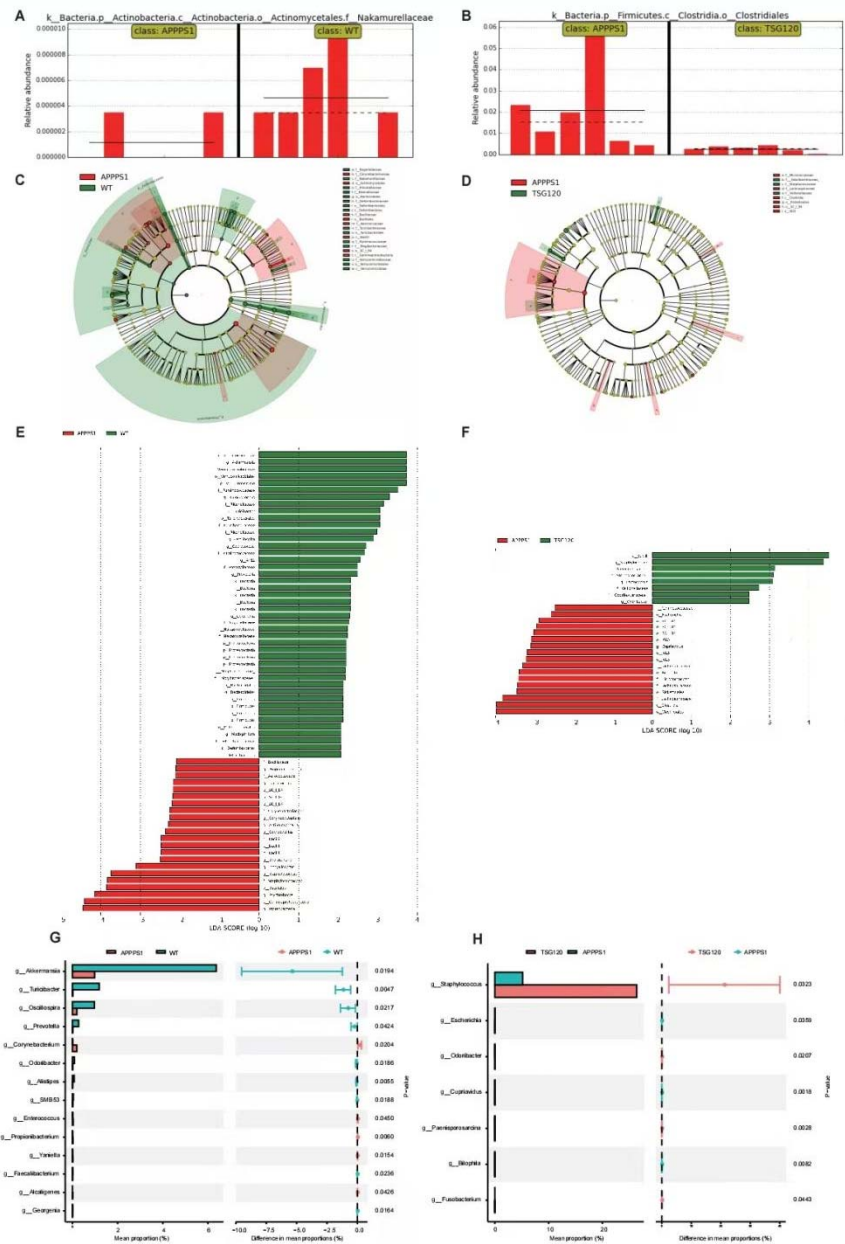


Fig. 7. TSG altered gut microbiota with different analysis by LefSe analysis. (A, B) Differential analysis of LefSe, showing the relative abundance of each group as APP/PS1 or APP/PS1+TSG 120 mg/kg group. (C, D) Cladogram. Different cycle layers in the cladogram radiate from the inside to outside to represent the seven taxonomic levels of the kingdom, phylum, class, order, family, genus, and species, respectively, and a species is represented by each dot. Yellow dots indicate no significant different between groups. Other colored dots represent significantly enriched microbiota in each group. (E, F) LDA score (log 10). (G, H) STAMP. t-test between APP/PS1 vs WT and TSG 120 vs APP/PS1, including mean proportion.

3.7 TSG changed the content of fecal SCFAs and colon morphology

Metabolites produced by the gut microbiota, notably SCFAs, vitamins, and neurotransmitters, are vital for gut-brain communication, impacting host neurophysiology and immunity. SCFAs exert their effects by reaching the brain via the circulatory system, where they participate in the regulation of memory and learning. To elucidate the regulatory effects of TSG on the intestinal microbiota, the levels of SCFAs in feces samples were examined, while these SCFAs were significantly reduced in feces of AD group (Fig. 8A to 8G). After

TSG intervention, the content of acetate, propionate and hexanoate were increased in the feces, but the contents of butyrate, isobutyrate, valerate and isovalerate were only slightly increased. A differential reduction in short-chain fatty acid levels—including acetate, butyrate, valerate, isovalerate, and more markedly, propionate, isobutyrate, and hexanoate—was observed in the AD group relative to WT controls. This deficit was ameliorated following TSG intervention.

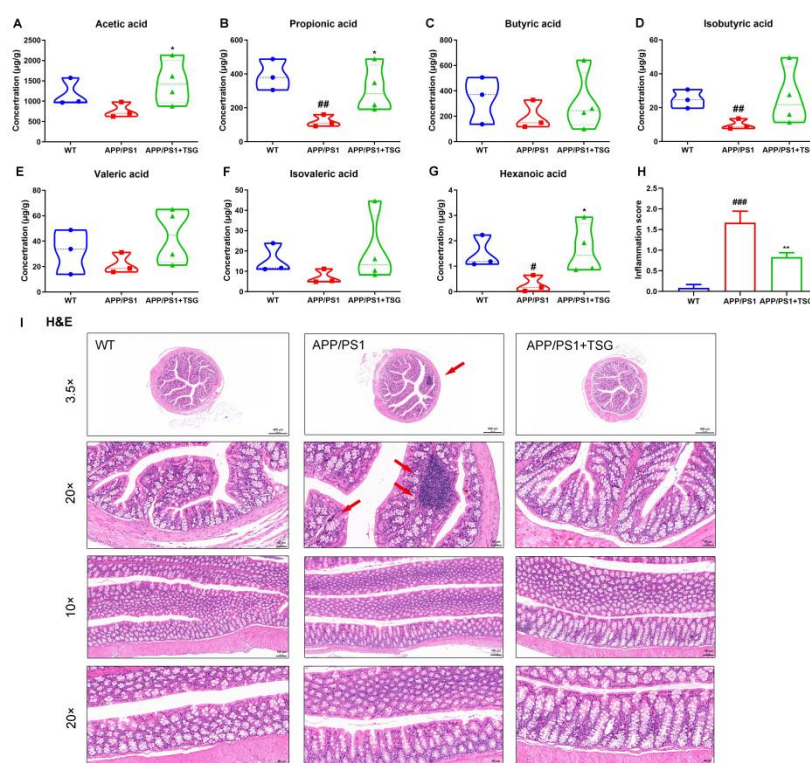


Fig. 8. Effects of TSG on the concentrations of SCFAs and intestinal tissue inflammation. (A) Acetic acid, (B) Propionic acid, (C) Butyric acid, (D) Isobutyric acid, (E) Valeric acid, (F) Isovaleric acid, and (G) Hexanoic acid. (H) Inflammation score and (I) Intestinal H&E staining to observe the effect of TSG on inflammation infiltration. Data are presented as mean $\pm$ SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control group; $^*P < 0.05$, vs APP/PS1 model group.

To evaluate the protective effect of TSG on the colon in AD mice, we performed H&E staining and histological examination. As shown in Fig. 8I, the colon structure in normal mice was intact, whereas the AD group exhibited significant inflammatory pathology, including mucosal neutrophil infiltration and submucosal edema. TSG treatment effectively ameliorated these pathological changes. The relative inflammation score was shown in Fig. 8H. These findings suggest that the neuroprotective effects of TSG may be mediated, in part, through the enhancement levels of SCFAs and the preservation of intestinal barrier integrity.

3.8 Correlation analysis of the relationship between behavioral outcomes, SCFAs and abundance of bacteria

Finally, we systematically performed the correlation analyses to elucidate the complex associations between four core gut microbial taxa and host SCFAs metabolism as well as multidimensional behavioral performance. In the analysis of microbial correlations with SCFAs (Fig. 9A), distinct positive and negative correlation patterns were observed between different microbial taxa and acetic acid, propionic acid, hexanoic acid, and butyric acid. Notably, *Lachnospiraceae* showed a strong positive correlation with butyric acid, and *Lactobacillales* with acetic acid, whereas *Staphylococcus* exhibited negative correlations with certain SCFAs,

suggesting that TSG treated microbial community structure significantly influence the synthesis and accumulation of intestinal SCFAs.

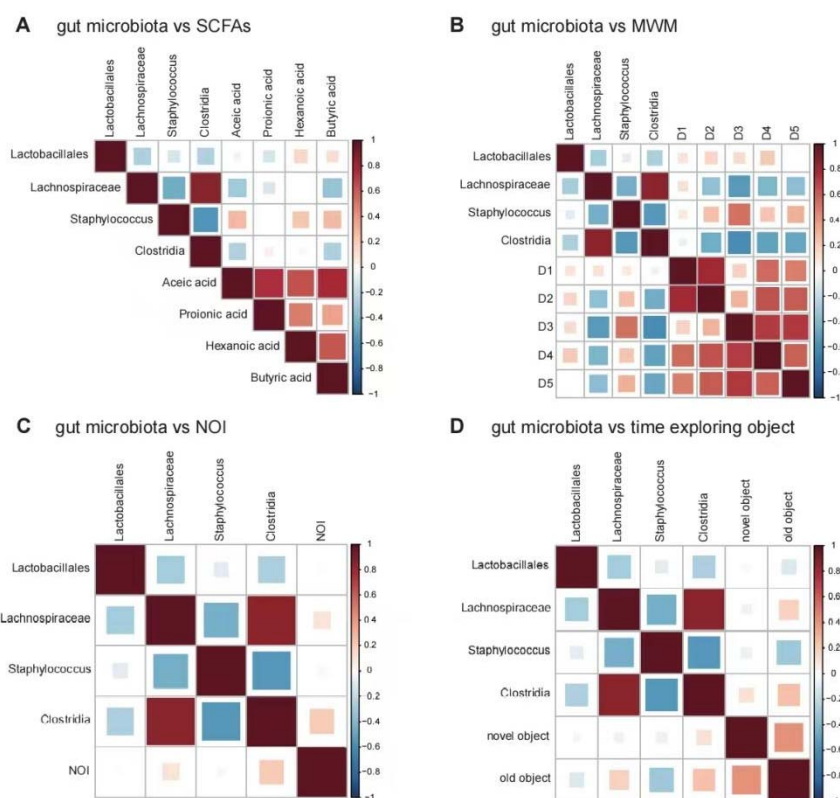


Fig. 9. The relationship between behavioral outcomes, SCFAs and the abundance of bacteria. Heatmap of (A) gut microbiota vs SCFAs, (B) gut microbiota vs MWM, (C) gut microbiota vs NOI, and (D) gut microbiota vs time exploring object. Red rectangle represents a positive correlation; blue rectangle indicates negative correlations. The color shows the strength of Pearson correlation coefficient ρ , and $P < 0.05$ was considered a significant correlation. Pearson correlation analysis, by calculating the correlation R value and P value. MWM, morris water maze; NOR, novel object recognition; NOI, novel recognition index.

At behavioral level, further analysis of dynamic relationship between microbial taxa and MWM (Fig. 9B) revealed that the correlation between microbial abundance and exploration time evolved over the course of five consecutive testing days. From D1 to D5, *Staphylococcus* exhibited significant positive correlation with MWM. Particularly in the later stages as D3 to D5, *Clostridia* showed a stable positive correlation, indicating that specific microbial taxa may be closely associated with cognitive recognition and spatial memory functions. Microbial composition was significantly correlated with NOI and time exploring object (Fig. 9C and 9D). Specifically, *Lachnospiraceae* and *Clostridia* correlated positively with NOI scores. In object recognition behavior test, *Lactobacillales* and *Lachnospiraceae* exhibited significant positive correlations with novel object exploration, but weak or negative correlations with familiar object exploration. Conversely, *Staphylococcus* correlated positively with familiar object exploration.

Collectively, these results demonstrate that TSG treated gut microbial composition is not only closely linked to SCFAs metabolites, but also regulate multiple cognitive and behavioral dimensions of the host, including novel object recognition, spatial learning, and exploratory behavior, potentially via metabolic or immune pathways within the gut-brain axis, providing important correlative evidence for underlying mechanisms.

4. Discussion

Previous studies have preliminarily established the therapeutic potential of TSG in AD models, particularly within the cortex and hippocampus of APP/PS1 mice [24–26, 28]. Building on this foundation, the present study employs an integrated multi-group design to comprehensively investigate the mechanisms of TSG in an AD mouse model. By applying 16S rDNA sequencing, we systematically evaluated the specific impact of TSG on the gut microbiota.

Accumulating evidence links pathogenic microbes, including bacteria, viruses, and fungi targeting the CNS, to AD risk. Such infections may exacerbate chronic neuroinflammation, thereby accelerating hallmark AD pathologies such as A β accumulation and synaptic degeneration [29]. Notably, bacterial infection can induce A β oligomerization, which possesses both antimicrobial and pathological properties, suggesting infection-induced amyloidosis as a potential AD trigger. Supporting this, APP/PS1 mice reared under germ-free conditions or treated with broad-spectrum antibiotics show reduced cerebral A β levels and plaque deposition [30]. Furthermore, modulating the gut microbiota via antibiotics or probiotics can alter learning and memory [31]. Therefore, we employed 16S rDNA amplicon sequencing to comprehensively analyze the gut microbiota in TSG-treated AD model mice. Our data indicate that TSG modulates both the abundance and diversity of gut microbiota. Given that high microbial diversity is a marker of host health while low diversity is a hallmark of dysbiosis linked to numerous diseases, these findings underscore the need for further interrogation of microbiota-immune pathways in AD development.

We further assessed species abundance and found that TSG treatment increased overall bacterial abundance. At the phylum level, TSG-administered APP/PS1 mice exhibited an increased abundance of *Bacteroidetes* and a reduction in *Firmicutes*. These two dominant phyla are crucial for maintaining host inflammatory and immune balance [32]. Notably, an elevated *Firmicutes/Bacteroidetes* ratio is often associated with a pro-inflammatory state and immune dysregulation in various disorders [33], consistent with our immunoassay data. At the genus level, TSG modulated the gut microbiota composition by enriching *Veillonella* and *Odoribacteraceae*, while reducing *Lachnospiraceae*. Many of these genera are known producers of SCFAs, such as acetate, propionate, and butyrate, which help maintain intestinal barrier integrity, glucose homeostasis, and immune regulation.

Previous studies have demonstrated that SCFAs can cross the blood-brain barrier, restore microglial morphology in germ-free mice, and mitigate stress-induced behavioral alterations, indicating their neuroprotective potential [34, 35]. Butyrate, for instance, enhances memory and neuroplasticity, and is primarily produced by genera such as *Clostridia*, *Lachnospiraceae*, and *Veillonellaceae*. Conversely, decreased levels of *Akkermansia muciniphila* and propionate have been observed in AD patients [36]. Oral propionate supplementation was shown to alleviate cognitive deficits in AD mice by modulating mitochondrial dynamics via G-protein coupled receptors (GPR41/43). Actually, SCFAs primarily exert their core functions by GPR41/43/109A and inhibiting histone deacetylases (HDACs). The GPR pathway mediates rapid responses in gut hormone secretion, immune regulation, and metabolic control, while HDAC inhibition, particularly by

butyrate, drives epigenetic regulation, promoting the expression of anti-inflammatory genes like *Foxp3* and maintaining intestinal homeostasis. These two pathways act synergistically, forming the key molecular basis through which SCFAs regulate host metabolism, immunity, and inflammatory responses. Herein, our findings suggested that TSG increases the levels of SCFAs in APP/PS1 mice provide a mechanistic explanation: TSG ameliorates neuroinflammation by enhancing the production, thereby establishing a causal link between TSG-induced gut microbiota remodeling and increased SCFAs.

Bacterial phyla such as *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* are commonly found in both AD patients and healthy controls [37]. Notably, the families *Burkholderiaceae* and *Staphylococcaceae* are enriched in AD brains. Research into gut-systemic inflammation links has shown that nearly 9% of microbiota variability correlates with IL-6 and IL-8 levels, and this association is slightly positive for *Proteobacteria* [38, 39]. Intriguingly, our data link the family *Odoribacteraceae* to TSG treatment. This finding is significant as *Odoribacteraceae* strains are known to synthesize isoalloLCA, a metabolite derived from centenarian gut isolates that suppresses pathobionts and potentially contributes to gut homeostasis.

In this study, we observed a significant increase in fecal SCFAs levels following TSG intervention. This result may stem from the multi-faceted regulatory effects of TSG on the gut microbiota: First, TSG act as a selective substrate promoting the proliferation of known SCFA-producing bacterial genera. Second, intermediate metabolites derived from TSG metabolism, together with the produced SCFAs, could lower the local pH in the colon, thereby further optimizing the ecological niche for acid-producing bacteria. Finally, the elevated SCFAs, especially acetic acid, propionic acid, and hexanoic acid, may in turn enhance intestinal barrier integrity, creating a favorable microenvironment for microbial homeostasis. These three aspects may work synergistically, forming a positive feedback loop.

Compared to other studies, the strength of this research lies in its well-defined bioactive compound and clear mechanistic entry point. It innovatively integrates a TCM monomer with modern microbiomics, laying a solid foundation for subsequent in-depth mechanistic investigations and drug development. Besides, similar studies on complex TCM formulations excel in their grand systems biology perspective and richness of data, perfectly illustrating the multi-target and holistic regulatory characteristics of herbal formulas [40]. They provide valuable resources for understanding the complex pathology of AD and the mode of action of multi-herbal interventions. The two approaches are highly complementary and together propel the advancement of this field.

Several limitations should be considered in this study. The reliance on APP/PS1 mice, which only model certain aspects of AD, limits direct translation to humans. Furthermore, while multi-faceted screening identified potential targets, subsequent validation is needed to confirm their roles. Our findings open promising avenues for future research that remain to be fully explored. Subsequent studies should prioritize in-depth investigation of the most characteristic data. Besides, the biomarkers identified may not be AD-specific, and their relevance to other conditions warrants further investigation. Comparing our data with those from other disease models could improve the interpretation of the results. Fourthly, due to the current

limitations of the conditions, we are unable to complete the FMT experiment. In the subsequent experiments, this part can be included for verification, making the research more comprehensive. Last, this study conducted solely in male APP/PS1 mice, identified a role for gut microbiota in AD pathology. This design presents a key limitation, as AD shows marked sex differences in patients, with greater female prevalence. Since sex hormones modulate both microbiota and neuro-immunity, our findings may not extend to females. Future work must include female models to compare sex-specific gut-brain axis mechanisms and to identify sex-dependent microbial biomarkers. Integrating such preclinical and clinical data is vital for developing personalized AD therapies.

5. Conclusion

Our study establishes that APP/PS1 mice develop chronic inflammation coupled with gut microbiota and metabolic dysregulation. Investigating the TCM-derived agent TSG, we discovered it alleviates cognitive impairment, restores gut microbial balance, and rectifies metabolic abnormalities. The protective mechanism of TSG involves modulation of inflammatory activity and enrichment of SCFAs-producing bacteria. These results not only elucidate a gut-brain axis mechanism in AD but also nominate TSG as a compelling therapeutic candidate with multi-faceted actions.

Funding declaration

Project was supported by grants from the National Natural Sciences Foundation of China (No. 82204645), Major Science and Technology Project of Henan Province (231100310200), Beijing Hospitals Authority Innovation Studio of Young Staff Funding Support (202317).

Ethics approval

All the experiments were approved by the Animal Care Committee of Capital Medical University (Certification No.: AEEI-2025-14). The authors made every effort to minimize the total number of all the mice.

Clinical trial number: not applicable.

Competing interests

The authors declare no conflict of interest..

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