

Gut Microbiota as the Gatekeeper: Oral Melatonin Breaks Ferroptosis to Rescue Alzheimer's Disease Brain and Colon in Iron Overload

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Abstract

Background: Gut-brain axis dysfunction contributes to Alzheimer's disease (AD) beyond amyloid/tau pathology, yet it remains unclear whether oral melatonin (MT) alleviates AD and gut comorbidities by modulating gut microbiota.

Objective: To investigate whether oral MT improves cognitive impairment, brain pathology, and gut comorbidities in AD mice and to elucidate microbiota-dependent mechanisms inhibiting oxidative stress and ferroptosis.

Methods: AD was induced in mice using $\text{AlCl}_3/\text{D-gal}$, followed by 6 weeks of oral melatonin (10 mg/kg/day). Cognitive function, oxidative stress, gut microbiota (16S rRNA), ferroptosis-related proteins, and fecal metabolites were assessed. Antibiotic intervention and fecal microbiota transplantation (FMT) validated microbiota dependency.

Results: Oral MT reshaped gut microbiota and metabolites, significantly improving cognitive deficits, reducing brain $\text{A}\beta_{1-42}$ and P-tau, alleviating colonic iron overload and dysfunction, and attenuating gut-brain oxidative stress (increased CAT/T-SOD, decreased MDA). MT repaired intestinal and blood-brain barriers and inhibited central ferroptosis via the Keap-1/Nrf-2/HO-1 axis. Antibiotic-induced dysbiosis exacerbated AD pathology. Notably, FMT from MT-treated donors partially recapitulated MT's protective effects, confirming that MT's anti-AD actions are partially microbiota-dependent.

Conclusion: Gut microbiota plays a key mediating role in MT's inhibition of ferroptosis against AD. Oral MT shows therapeutic potential for managing AD-related gut-brain comorbidity, supporting microbiota-based AD treatment strategies.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline. By 2050, the global prevalence is projected to reach 150 million [1], placing a heavy economic and caregiving burden on society and healthcare systems. The two classic histopathological hallmarks of AD are extracellular senile plaques formed by the aggregation of β -amyloid ($\text{A}\beta$) and intracellular neurofibrillary tangles composed of aggregates of hyperphosphorylated tau protein (P-Tau) [2]. However, in addition to $\text{A}\beta$ plaques and tangles, AD is associated with a variety of biochemical and pathological changes that often manifest before the appearance of plaques and tangles, including disturbances in calcium homeostasis, abnormalities in cholesterol and phospholipid metabolism, and alterations in mitochondrial dynamics [3–5]. Extensive research indicates that AD is not an isolated brain disease; through complex brain-body interactions, it profoundly affects the function and homeostasis of multiple organ systems throughout the body.

Dysregulation of communication between organ systems is increasingly recognized as a key factor in the onset and progression of AD. Recent studies have consistently revealed that information exchange

across multiple systems—including the gut-brain axis, brain-adipose tissue axis, brain-liver axis, brain-immune system axis, and brain-muscle axis—plays a significant role in AD. The peripheral metabolic system modulates neuroinflammation, protein misfolding, and neuronal dysfunction by releasing cytokines, adipokines, growth factors, and other soluble molecules [6]. Liu et al. found that dysfunction of the neuroimmune axis can disrupt central nervous system (CNS) homeostasis, inducing enhanced neuroinflammation, oxidative stress, and mitochondrial dysfunction, ultimately leading to neuronal dysfunction, damage, and even death. Furthermore, alterations in blood-brain barrier integrity facilitate the entry of peripheral immune cells and inflammatory mediators into the CNS, further exacerbating the pathological cascade [7]. Under pathological conditions, hepatic dysfunction and the resulting accumulation of toxic metabolites can exacerbate neuroinflammation in the CNS and accelerate neurodegenerative progression [8]. Concurrently, gut microbiota dysbiosis in AD can drive immune activation, disrupt protective barriers, and alter neuroregulatory signaling [9]. Dysregulation of these inter-organ pathways collectively contributes to the systemic manifestations of AD; therefore, the complex bidirectional interactions between the brain and peripheral organs warrant further attention.

In recent years, the influence of the gut microbiome on the brain and the mechanisms of gut-brain communication have become a hot topic of research. Existing studies have confirmed that patients with AD often exhibit significant gut dysbiosis and gastrointestinal dysfunction [10]. Multiple research groups have reported that this dysbiosis is closely associated with neuroinflammation, β -amyloid aggregation, and increased oxidative stress during the course of AD [11]. The gut microbiome consists of trillions of microorganisms, and its homeostasis plays a critical role in regulating host physiological activities and autoimmune diseases. Extensive research indicates that the gut microbiota exerts a significant regulatory effect on CNS function by participating in bidirectional gut-brain communication; simultaneously, the CNS reshapes the gut microbiome through neural, endocrine, immune, and metabolic pathways. Together, they form a complex “microbiota-gut-brain axis” regulatory network that collaboratively maintains brain homeostasis [12]. Short-chain fatty acids (SCFAs), as key metabolites produced by gut bacteria through the fermentation of dietary fiber, not only participate in the regulation of digestion, immune responses, and energy metabolism but also possess significant anti-inflammatory properties. They enhance intestinal barrier integrity, thereby contributing to the maintenance of host homeostasis [13]. Based on this, integrating the mechanisms by which gut microbiota influence brain function into the theoretical framework of drug action holds promise for providing new insights into the development of novel therapeutic strategies for AD.

Melatonin (MT) is a multifunctional neurohormone secreted by the pineal gland. As an endogenous antioxidant and free radical scavenger, it plays a role in regulating various physiological functions. It exerts powerful cytoprotective effects by preventing lipid peroxidation, oxidative protein damage, and oxidative DNA damage, as well as by regulating cellular energy metabolism. As research into the functions of MT has deepened, it has been shown to possess a wide range of biological activities beyond the regulation of circadian rhythms, including effects on reproduction, immune responses, and antioxidant and anti-aging properties, making it a crucial molecule for maintaining health [14–16]. Recent studies have highlighted the therapeutic potential of MT in neurodegenerative diseases. Clinical

observations have revealed that patients with AD often exhibit reduced nocturnal MT secretion and severe sleep disturbances [17]. Exogenous melatonin supplementation has been shown to alleviate Tau hyperphosphorylation and reduce A β plaque formation [18]. Collectively, these findings suggest that MT, as a multi-target therapeutic molecule, offers a novel strategy for intervening in the pathological progression of AD. It is worth noting that establishing a more comprehensive “systemic Alzheimer’s disease” perspective from the “brain-body connection” angle is of great significance for the management of AD-gut-brain comorbidity, improving quality of life, and delaying functional decline. However, there are currently no published reports on whether MT ingested via the gastrointestinal tract alleviates AD pathology by regulating the gut microbiota and exerting antioxidant effects.

To better elucidate potential viable options for improving brain health through medication, exploring the relationships between biological systems may be an effective approach to addressing disease. Based on these considerations, this study aims to comprehensively employ viable options and explore the relationships between biological systems as a potential effective means of addressing disease. Based on the above considerations, this study aims to comprehensively employ techniques such as behavioral science, pathophysiology, high-throughput 16S rRNA gene sequencing, and molecular biology to systematically evaluate the potential of MT to reshape the gut microbiota and microbial metabolism, as well as its effects on repairing the intestinal barrier and alleviating oxidative stress. Furthermore, the study will thoroughly investigate its role in regulating iron homeostasis and inhibiting ferroptosis, elucidate the mechanisms by which gut microbiota mediate MT’s antioxidant effects and ferroptosis inhibition in AD, and ultimately reveal its ability to improve cognitive dysfunction and pathological damage associated with AD, with the aim of providing promising strategies for the prevention, diagnosis, and treatment of AD.

2. Materials and Methods

2.1 Reagents

D-gal (McLean, China), anhydrous aluminum chloride (AlCl₃, McLean, China), melatonin (McLean, China), EDTA-2K anticoagulant (Solebao, China), high-performance RIPA lysis buffer (Solebao, China), 5× SWE rapid high-resolution electrophoresis buffer (Seville, China), sodium acetate (McLean, China), neutral formalin fixative (Seville, China), BCA protein concentration assay kit (Bioyuntian, China), 12% SDS-PAGE gel (Sikejie, China), ECL detection kit (Biosharp, China), PVDF membrane (Merck Millipore, Germany), skim milk powder (Biofroxx, Germany), sodium acetate (McLean, China), gentamicin sulfate (Fuan Pharmaceutical Group Yantai Zhichu Pharmaceutical Co., Ltd., China), furazolidone enteric-coated tablets (Tianjin Lisheng Pharmaceutical Co., Ltd., China), metronidazole tablets (Beijing Fuyuan Pharmaceutical Co., Ltd., China), ampicillin (Hong Kong Federal Pharmaceutical Factory Co., Ltd., China), and other reagents.

2.2 Animals

All animal experimental protocols in this study were approved by the Ethics Review Committee of Xinhua Hospital Affiliated to Dalian University (Approval No.: k2024-002). Six-week-old male ICR mice used in the experiments were purchased from Liaoning Changsheng Biotechnology Co., Ltd. All animals were housed in a specific-pathogen-free (SPF) environment at a temperature of $23 \pm 2^{\circ}\text{C}$, with a relative humidity of 50% – 60%, and maintained on a 12 hour light-dark cycle. During the experiment, mice had free access to standard chow and drinking water. Prior to the experiment, the animals were acclimatized in the housing facility for 1 week.

2.3 Experimental Design

In the first part of this study, mice were randomly assigned to three groups ($n = 12$): a control group, a model group, and a MT group. The remaining groups received intraperitoneal injections of AlCl_3 (40 mg/kg) and D-gal (100 mg/kg) during weeks 1–3; during weeks 4–6, the dosage was adjusted to AlCl_3 (20 mg/kg) and D-gal (100 mg/kg). Treatment was initiated concurrently with model induction, and the MT group was administered melatonin (10 mg/kg) via oral gavage for six weeks. After the behavioral assessments were completed, all mice were euthanized, and samples were collected for analysis. The melatonin dosage employed in this study has been extensively adopted in previous studies. Accordingly, our group performed a pilot experiment to validate its efficacy, and the results demonstrated that this dosage markedly ameliorated cognitive deficits and relevant pathological features in AD-like model mice. Consequently, this dosage was selected for the subsequent formal experiments [19, 20].

In Part 2, mice were randomly assigned to three groups: Control, Model, and ABX _ Model (antibiotic cocktail intervention combined with the Model group) ($n = 12$). The modeling method for the AD model mice was consistent with that in Part 1. Additionally, mice in the ABX _ Model group were administered the ABX antibiotic mixture via oral gavage for 7 consecutive days. The mixture consisted of gentamicin sulfate (5 mg/kg), furazolidone enteric-coated tablets (25 mg/kg), metronidazole tablets (100 mg/kg), and fluconazole tablets (1 mg/kg), and were provided with drinking water containing ampicillin (1 g/L). After the behavioral assessments were completed, all mice were euthanized, and samples were collected for analysis.

In Part 3, mice were randomly divided into the Control group, Model group, MT group, and ABX + MT group ($n = 12$). The method for establishing the AD model in mice was consistent with that described in Part 1. Mice in the MT and ABX + MT groups were administered melatonin (10 mg/kg) via oral gavage for 6 weeks. Concurrently, mice in the ABX + MT group were administered a mixture of ABX antibiotics via oral gavage for 7 consecutive days, starting one week prior. After the behavioral assessments were completed, all mice were euthanized, and samples were collected for analysis.

2.4 Morris Water Maze Experiment

In the spatial navigation experiment, mice in each group underwent four training sessions daily, entering the water from the midpoint of each quadrant. The time required to locate the platform within 60 seconds was recorded; the time from entry into the water to reaching the platform was defined as the escape latency. If the platform was not located, the session was recorded as 60 seconds, and the mouse

was guided onto the platform to remain there for 15 seconds. On day 6, the platform was removed, and a 60-second spatial exploration test was conducted with mice entering the water from the second quadrant. A computer tracking system (63031, Shenzhen Ruowode Life Technology Co., Ltd., China) was used to track the animals' movements.

2.5 Y-Maze Experiment

The Y-Maze assesses working memory by measuring the spontaneous alternation rate. Mice are placed at the end of one arm leading to the center of the Y-maze (63006, Shenzhen Ruowode Life Science Technology Co., Ltd., China) and allowed to explore freely for 5 minutes. Spontaneous alternation rate = (number of correct alternations) / (total number of alternations – 2) × 100%.

2.6 Individual Nest-Building Experiment

One mouse was selected from each group and housed in a cage containing a 7-cm square cotton pad. Nest-building behavior was scored for each group at 12, 24, and 48 hours. Nest-building behavior was scored on a scale of 1–5: 1 point for a complete nest with few gnaw marks; 2 points for a nest that was chewed into fragments and scattered; 3 points for a completely disorganized nest with no distinct shape; 4 points: preliminary nest shape with defects; 5 points: typical nest with varying heights. The final nesting score for each group of mice was calculated as the average of three independent evaluations conducted by three experimenters.

2.7 Tail Suspension Test

The tail suspension test uses an opaque rectangular test chamber (30 cm long × 30 cm wide × 70 cm high) with a hook (3 cm long) attached to the top. At the start of the experiment, the tip of the mouse's tail is secured with adhesive tape, and the mouse is suspended from the test chamber with its head 30 cm above the ground. The mouse is allowed to move freely for a total of 6 minutes. After a 2-minute acclimatization period, the duration of complete limb immobility during the final 4 minutes is recorded as the immobility time. After the experiment, the apparatus is wiped with 75% alcohol swabs to remove any residual odors or fluids from the previous mouse.

2.8 Novel Object Recognition Experiment

Rodents have an innate preference for novel objects. The apparatus consisted of a box made of acrylic (25 cm × 25 cm × 25 cm) placed in a separate room. We recorded and analyzed videos of the animals' behavior. The experiment consisted of three phases: habituation, training, and testing. During training, mice were allowed to explore two identical objects for 10 minutes. Twenty-four hours later, one of the two familiar training objects was replaced with a novel test object. If a mouse recognized the familiar object, it would spend most of its time on the novel object, reflecting its intact recognition memory. A recognition index for the novel object was calculated to assess the impact on the mice's recognition memory.

2.9 16S rRNA Sequencing

To ensure the accuracy and reliability of the sequencing data, strict quality control measures were implemented at every stage of the experiment. Fresh mouse fecal samples were collected in sterile tubes, immediately flash-frozen in liquid nitrogen, and stored at -80°C. Total DNA was extracted using a fecal genomic DNA extraction kit, and a short-fragment library targeting the V3-V4 hypervariable regions of the 16S rRNA gene was constructed. Bisequenced sequencing was performed using the Illumina NovaSeq platform. After quality control, assembly, and filtering, the raw sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity and into amplicon sequence variants (ASVs) based on 100% similarity. High-quality sequences were selected for subsequent bioinformatics analysis.

2.10 LC-MS/MS Analysis of Metabolites

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (SCIEX, QTRAP 6500+) was employed to quantitatively detect SCFAs, lactic acid, and deoxycholic acid in fecal samples. The fecal sample preparation method followed the procedure reported in [21], and the detection conditions were based on the literature reported in [22].

2.11 Hematoxylin and Eosin (HE) Staining

After euthanizing the mice, the brain and colon tissues were harvested and fixed in neutral formalin for 48 hours. The tissues were then fixed, dehydrated, embedded in paraffin, and sectioned. The sections were dewaxed in a water-based, eco-friendly dewaxing solution (I and II) for 15 minutes each, followed by anhydrous ethanol (I and II) for 5 minutes each, 75% ethanol for 5 minutes, and then rinsed with tap water. The sections were treated with a high-definition staining pretreatment solution for 1 minute. Hematoxylin staining was performed for 3 minutes, followed by differentiation for 3–5 seconds, re-blueing for 3–6 seconds, and rinsing under running water. Dehydrate in 95% ethanol for 1 min, then stain with eosin for 15 s. Successively immerse in anhydrous ethanol I, II, and III for 1 min each, n-butanol I and II for 1 min each, and xylene I and II for 1 min each until clear, then mount with neutral gum. After frozen sections return to room temperature, fix in acetone for 1 min. Observe under a light microscope; capture and analyze images using CaseViewer software.

2.12 Histopathological Evaluation of Colonic Tissue

The histological scoring criteria are as follows: Inflammation severity (0–3 points: none, mild, moderate, severe); Depth of injury (0–3 points: none, mucosal layer, mucosa and submucosa, transmural); Crypt damage (0–4 points: none, damage to the basal third, damage to the basal two-thirds, only surface epithelium intact, complete loss of crypts and epithelium); Area of colonic involvement (0–4 points: none, 1–25%, 26–50%, 51–75%, > 75%). The total score for each sample is the sum of the four scores (0–14 points), which comprehensively reflects the degree of colonic inflammation and damage.

2.13 Immunofluorescence and Immunohistochemistry

After fixation for 48 hours, the mouse brain and colon tissues were sequentially dehydrated, embedded in paraffin, and sectioned. Primary antibodies were added and incubated overnight at 4°C. After washing

three times with PBS, the corresponding secondary antibodies were added and incubated at room temperature, followed by another wash. Finally, the sections were examined under a microscope, and images were captured and analyzed. Primary antibodies used included: A β (1:2500), NeuN (1:5000), IBA-1 (1:1500), DJ-1 (1:1000), Gpx-4 (1:2500), Claudin-5 (1:500), Occludin (1:500), ZO-1 (1:200), MUC-2 (1:500), and MCT-1 (1:200), all from ServiceBio (Wuhan, China).

2.14 Biochemical Assays

Commercial kits: MDA kit (Nanjing Jiancheng, China), CAT kit (Nanjing Jiancheng, China), T-SOD kit (Nanjing Jiancheng, China), GSH kit (Solabio, China), and tissue iron kit (Nanjing Jiancheng, China). Mouse brain and colon tissues were processed and analyzed according to the manufacturer's instructions for use.

2.15 Western Blot

Total protein was extracted from brain tissue and homogenized with lysis buffer at a 1:10 (w/v) ratio. After determining the protein concentration using the BCA method, equal amounts of 40 μ g of protein were separated by 12% SDS-PAGE and transferred to a 0.22 μ m PVDF membrane. To block nonspecific binding, the membrane was blocked with 5% skim milk at room temperature for 1 hour, followed by incubation with the primary antibody overnight at 4°C and the corresponding secondary antibody for 2 hours at room temperature. Finally, the membrane was visualized using a chemiluminescence assay and analyzed using ImageJ software. Primary antibodies: anti-A β ₁₋₄₂ (1:1000), anti-P-Tau (1:1000), anti-Keap-1 (1:1000), anti-Nrf-2 (1:1000), anti-GAPDH (1:10,000), and anti-MCT-1 (1:1000) from BLOSS (Beijing, China); anti-NeuN (1:1000), anti-HO-1 (1:1000), and anti-DJ-1 (1:1000) were obtained from ServiceBio (Wuhan, China); anti-IREB2 (1:1000) and anti-FTH (1:2000) were obtained from Signalway Antibody LLC (USA).

2.16 Fecal Microbiota Transplantation (FMT) Experiment

Mice were randomly assigned to Control, Model, and FMT_MT groups (n = 12), with the latter receiving fecal microbiota transplantation (FMT) from donor mice that had been pretreated with MT. The FMT_MT group was first administered ABX for one week in advance, following the method described in Section 2. Subsequently, all three groups underwent parallel treatment. The FMT_MT group was administered 0.3 mL of a fresh, processed fecal microbiota suspension via gavage daily for 6 weeks. The fecal microbiota suspension was prepared as follows: Feces were collected 24 hours after MT administration, mixed with sterile PBS at a ratio of 100 mg:1 mL, vortexed, allowed to stand for 30 minutes, and then centrifuged at 3,500 rpm for 10 minutes. The supernatant was immediately administered via gavage. The Model group was established as described in Section 2.3. All mice were euthanized after completing the behavioral tests in sequence, and samples were collected for subsequent analysis.

2.17 MT Residue Detection

Accurately weigh 10 mg of MT, transfer it to a 10-mL volumetric flask, add methanol, and dilute to a final volume of 10 mL. Analyze using high-performance liquid chromatography (HPLC) and record the

retention times of the standard samples. After collecting mouse feces 24 hours following MT administration, euthanize the mice, dissect them, and remove the brain tissue. Cut 100 mg of brain tissue into pieces, place in a tissue homogenization tube, add 1 mL of methanol, and homogenize for 5 minutes using an ultrasonic cell disruptor. Centrifuge (2000 ×rpm, 5 minutes) and collect 800 µL of the supernatant. Concentrate the supernatant using a vacuum concentrator, redissolve in 100 µL of methanol, and use HPLC to determine whether MT is present in the brain tissue. Chromatographic conditions are as follows: Column: C18 column (5 µm, 4.6 × 250 µm); Injection volume: 20 µL; Detection wavelength: 222 nm; Isocratic elution; Mobile phase: methanol: 0.2% phosphoric acid aqueous solution (v: v) = 70:30; Flow rate: 1 mL/min.

2.18 Statistical Analysis

All collected data were analyzed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). All results are expressed as mean ± standard error (x ± SEM). Differences between two or more groups were analyzed using one-way analysis of variance (ANOVA). T-tests (two-tailed) were used to determine differences between two groups. A P-value < 0.05 was considered statistically significant, where: *P < 0.05, **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. Model group and MT group; and ns indicates no significant difference.

3 Results

3.1 MT Alleviates Cognitive Impairment in Mice with an AD Model

To determine the effect of MT on cognitive impairment in AD model mice, behavioral experiments were conducted to evaluate the impact of MT intervention on spatial learning and memory abilities in these mice (Fig. 1A). Monitoring of body weight across the groups revealed that body weight in the Control group continued to increase over six weeks, while the average body weight in the Model group was lower (P < 0.01). In contrast, body weight in the MT group increased compared to that in the Model group (P > 0.05) (Fig. 1B).

The Y-maze is commonly used to assess short-term spatial memory in animals. Compared with the Control group, the Model group exhibited a significantly reduced spontaneous alternation rate (P < 0.01), whereas the MT group showed a significantly higher alternation rate than the Model group (P < 0.01) (Fig. 1C), indicating that MT alleviates short-term memory impairments induced by AlCl₃/D-gal.

The TST is a classic test for assessing depression-like behavior, and an increase in cumulative immobility time serves as an indicator of despair. TST results showed that the immobility time of mice in the Model group was significantly longer than that of the Control group (P < 0.01), whereas MT intervention significantly shortened the immobility time in AD model mice (P < 0.01) (Fig. 1D), indicating that depression-like behavior in the model mice was improved.

The Morris water maze was used to assess spatial learning and long-term memory. From Day 3 of training, the escape latency in the Model group was significantly longer than that in the Control group ($P < 0.05$). By Day 5, the MT group showed significantly shorter latency compared with the Model group ($P < 0.01$) (Fig. 1E), indicating that MT improved learning and memory in AD mice. Representative thermal heatmaps from the spatial exploration test showed that Control mice quickly located the platform, Model mice swam aimlessly along the periphery, and MT mice relatively quickly found the platform in the target quadrant (Fig. 1F). No significant differences in swimming speed were observed among the three groups (Fig. 1G). Compared with the Control group, the Model group exhibited typical cognitive impairments, including significantly longer latency to first platform arrival, reduced time in the target quadrant, and fewer platform crossings and target quadrant entries (all $P < 0.01$). In contrast, MT intervention significantly reversed these deficits, as shown by shorter first-arrival latency, increased target quadrant duration, and greater platform crossings and quadrant entries (all $P < 0.01$) (Fig. 1H-K).

The nest-building test is a behavioral assay used to assess mice's self-care abilities and emotional state, reflecting their adaptability to environmental changes and basic survival skills. We conducted an individual nest-building test, which revealed that mice in the Control group exhibited distinct nest-building behavior as early as 12 h, whereas the nest-building scores of the Model group were significantly lower than those of the Control group ($P < 0.05$), indicating that $\text{AlCl}_3/\text{D-gal}$ induction caused deficits in nest-building ability. The 48-h nest-building results showed that the nest-building scores of mice in the MT intervention group were significantly higher than those of the Model group ($P < 0.05$) (Fig. 1L-M), indicating that MT intervention can enhance the basic survival skills of AD model mice. Overall, MT can improve short-term memory deficits, depression-like behavior, spatial learning impairments, and basic survival skills in AD model mice, thereby alleviating cognitive dysfunction in these mice.

3.2 MT simultaneously alleviates neuronal damage and intestinal dysfunction in mice with an AD model

To further elucidate the neuroprotective effects of MT on AD model mice, HE staining was performed on the dentate gyrus (DG) region of the brain tissue from mice in each group. The results showed that, compared with the Control group, mice in the Model group exhibited a reduced number of neurons, disordered arrangement, darker nuclear staining, and cell body atrophy with blurred boundaries. Compared with the Model group, the MT intervention reduced the number of damaged neurons and restored neuronal morphology and structure, as evidenced by more orderly cell arrangement and reduced nuclear condensation. Although the ABX + MT intervention reduced neuronal loss, it was less effective than the MT group in restoring brain tissue damage in AD model mice (Fig. 2A).

Excessive phosphorylation of tau protein and deposition of $\text{A}\beta$ protein are key pathological features in the progression of AD [23]. NeuN, a marker of mature neurons, shows reduced expression that may adversely affect memory function [24]. Western blot (WB) results demonstrated that, compared with the Control group, the expression levels of P-Tau and $\text{A}\beta_{1-42}$ in the brain tissue of mice in the Model group

were significantly increased ($P < 0.05$, $P < 0.01$). In the MT group, these elevated levels of P-Tau and $A\beta_{1-42}$ were attenuated ($P < 0.05$, $P < 0.01$). The ABX + MT group downregulated $A\beta_{1-42}$ expression ($P < 0.01$) but did not significantly affect P-Tau expression compared with the Model group ($P > 0.05$). No significant differences in P-Tau or $A\beta_{1-42}$ expression were observed between the MT group and the ABX + MT group (both $P > 0.05$) (Fig. 2B-D). Regarding NeuN protein expression, the Model group exhibited a marked reduction compared with the Control group ($P < 0.01$). The MT group upregulated NeuN expression relative to the Model group ($P < 0.05$), whereas the ABX + MT group failed to reverse the low NeuN expression seen in the Model group ($P > 0.05$). Moreover, NeuN expression in the ABX + MT group was significantly lower than that in the MT group ($P < 0.01$) (Fig. 2E). These findings were further corroborated by immunofluorescence staining; despite minor variations in individual statistical significance, the overall trends were consistent. In addition, immunofluorescence results revealed that IBA-1 levels in the Model group were significantly higher than those in the Control group ($P < 0.01$). The MT intervention group markedly reduced IBA-1 expression ($P < 0.01$), while the ABX + MT group showed no significant difference in IBA-1 levels compared with the Model group ($P > 0.05$). Furthermore, no significant difference in IBA-1 expression was observed between the MT group and the ABX + MT group ($P > 0.05$) (Fig. 2F-I).

Inflammatory bowel disease has increasingly been shown to be associated with the progression of neurodegenerative diseases, particularly AD [25]. Therefore, we further investigated the effects of MT on the structural and functional characteristics of the colon in AD model mice. Colon length is typically closely associated with the severity of intestinal inflammation. We found that, compared with the Control group, the colon length in AD model mice was significantly shorter ($P < 0.01$), reflecting intestinal inflammation and functional dysfunction. Following intervention with MT and ABX + MT, the trend of shortened colon length in AD mice was significantly reversed ($P < 0.01$ for both), but no significant difference was observed between the MT and ABX + MT groups ($P > 0.05$) (Fig. 2J-K). Colonic histopathological scoring results showed that the Model group had significantly higher scores than the Control group ($P < 0.01$). Compared with the Model group, the MT group exhibited significantly lower scores ($P < 0.05$), while the ABX + MT group showed a trend toward lower scores but no significant difference ($P > 0.05$). Furthermore, there was no significant difference between the MT and ABX + MT groups ($P > 0.05$) (Fig. 2L). Hematoxylin and eosin (HE) staining of colonic tissue further confirmed this improvement. Compared with the Control group, the colons of mice in the Model group exhibited disorganized cellular arrangement, a reduction in goblet cells, abnormal crypt structure, and extensive inflammatory cell infiltration. Compared to the Model group, the colonic gland morphology in the MT and ABX + MT groups was restored, with an increase in goblet cell numbers and a reduction in inflammatory cell infiltration. However, there were differences in the degree of improvement between the two groups: the MT group exhibited more significant restoration of colonic tissue structure, with more orderly gland arrangement and a more pronounced increase in goblet cell numbers. In contrast, the reparative effect on colonic tissue in the ABX + MT group was relatively limited compared to that of the MT group (Fig. 2M).

The above results indicate that MT can reduce levels of pathological AD-associated proteins and alleviate neuronal damage in AD. Additionally, MT effectively restores colon length and glandular structure. The combined ABX intervention partially attenuated the neuroprotective effects of MT on the central nervous system and its tissue-repairing effects on peripheral colon tissue, suggesting that an appropriate gut microbiota composition contributes to the neuroprotective effects of MT in AD.

3.3 MT-Induced Dysbiosis of the Gut Microbiota and Microbial Metabolism in AD Model Mice

Disruption of the gut microbiota is closely associated with the onset and progression of AD. How MT influences changes in gut microbiota structure and thereby alleviates cognitive impairment in AD is an important area requiring further investigation. 16S rRNA sequencing results showed that 293, 351, and 346 ASVs were detected in the Control, Model, and MT groups, respectively (Fig. 3A). PLS-DA results demonstrated a trend of intra-group clustering and inter-group separation in the overall structure of the gut microbiota across the three groups. The MT group was closely aligned with the Control group, and both were distinct from the Model group (Fig. 3B). Alpha diversity analysis of gut microbiota in fecal samples from each group revealed no significant differences in ACE, Chao-1, Shannon, or Simpson indices across groups ($P > 0.05$ for all) (Fig. 3C). In the inter-sample distance matrix heatmap at the ASV level, smaller circles and lighter colors indicate lower beta diversity values and smaller diversity differences between samples. The results show that, compared to the Control group, the Model group exhibits lighter colors and smaller circles, indicating lower beta diversity in the Model group. Following MT intervention, the sample colors darkened (Fig. 3D), indicating that beta diversity was restored, confirming that MT reshaped the overall structure of the gut microbiota.

Quantitative analysis revealed that, at the phylum level, the relative abundances of the major gut microbiota in the Control group were 83.8701% for *Firmicutes*, 6.7347% for *Proteobacteria*, 0.0398% for *Cyanobacteria*, 0.1699% for *Actinobacteriota*, and 0.0028% for *Actinobacteria*, while those in the Model group were 84.6402%, 1.3753%, 0.0026%, 2.6328%, and 0.0013%, respectively. In the MT intervention group, the relative abundances of these microbial groups were 3.0914%, 58.1877%, 11.8654%, 7.0537%, and 7.4777% (Fig. 3E). Comparative analysis revealed that MT intervention significantly reduced the relative abundance of *Firmicutes* in the gut microbiota and increased the relative abundance of *Proteobacteria*, *Cyanobacteria*, *Actinobacteriota*, and *Actinobacteria*.

By constructing a phylogenetic tree, we reconstructed the relationships among different populations and identified the most influential taxonomic units following MT intervention. Using LEfSe and LDA scores, we assessed the contribution of each taxonomic unit's relative abundance to intergroup differences and screened for significantly different microbial groups. Using a threshold of LDA score > 4 and $P < 0.05$, a total of 113 differential biomarkers were identified across the Control, Model, and MT groups, of which 51 were enriched in the MT group. Additionally, ternary plots illustrated the distribution of core microorganisms at the class level across the groups, as shown in Fig. 3F-H. Furthermore, differential microbial analysis at the phylum, order, family, and genus levels revealed that, compared with the Model

group, MT intervention increased the relative abundance of the SCFA-producing *Bacteroidota* phylum from 0.032 to 0.064, a 1.5-fold increase, but this was not statistically significant ($P > 0.05$). At the same time, MT intervention significantly downregulated the relative abundances of the order *Erysipelotrichales*, the family *Erysipelotrichaceae*, and the family *Oscillospiraceae* ($P < 0.05$, $P < 0.01$, $P < 0.01$). Furthermore, MT intervention suppressed the abundance of multiple bacterial genera, including *Turicibacter*, *Dialister*, *Lachnospira*, *Fournierella*, and *Olsenella* ($P > 0.05$, $P > 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.05$) (Fig. 3I). These results indicate that MT can reshape the structural imbalance of the gut microbiota in AD, upregulate the SCFA-producing *Bacteroidota* phylum, and suppress the proliferation of inflammation-associated potentially harmful genera (such as *Dialister*, *Turicibacter*, and *Oscillospiraceae*).

LC-MS/MS was used to measure metabolite levels in feces. The results showed that, compared with the Control group, the total SCFA content in the feces of mice in the Model group was significantly reduced ($P < 0.05$), whereas the total SCFA content in the feces of the MT intervention group increased ($P < 0.01$) (Fig. 3J). As key metabolites of the gut microbiota, SCFAs play a vital role in maintaining host health. Furthermore, lactate levels in the Model group were significantly lower than those in the Control group ($P < 0.05$), and MT intervention reversed the reduction in fecal lactate levels in AD model mice ($P < 0.01$). Concurrently, compared with the Control group, deoxycholate levels in the feces of the Model group were abnormally elevated ($P < 0.01$), and MT intervention further increased these levels ($P < 0.01$) (Fig. 3K-L). These results confirm that MT can reshape the gut microbiota and regulate the levels of microbial metabolites, providing preliminary evidence that MT may exert its anti-AD effects through the microbiota-gut-brain axis.

3.4 Combined ABX treatment reduces the alleviating effect of MT on cognitive dysfunction in AD model mice

Dysbiosis of the gut microbiota has been recognized as a key feature of the pathophysiological process of AD. This dysbiosis is not only present in AD patients but also emerges during the mild cognitive impairment (MCI) stage, suggesting that it may serve as an early indicator of AD progression [26]. To evaluate whether gut microbiota mediate the effect of MT on cognitive impairment in AD, this study introduced an ABX + MT intervention group (Fig. 4A). By week 5, both MT and ABX + MT groups had non-significantly higher weights than the Model group ($P > 0.05$), with no difference between the two (Fig. 4B). Compared with the Control group, the Model group exhibited a reduced spontaneous alternation rate in the Y-maze and prolonged immobility time in the tail suspension test (both $P < 0.01$). Both MT and ABX + MT significantly improved these deficits ($P < 0.01$), with no significant differences between them ($P > 0.05$) (Fig. 4C-D).

In the Morris water maze (Day 5), Both MT and ABX + MT significantly reduced escape latency ($P < 0.01$), with no difference between them ($P > 0.05$) (Fig. 4E). On Day 6 (spatial exploration), MT significantly shortened the distance to first reach the platform ($P < 0.01$), whereas ABX + MT did not ($P > 0.05$), and ABX + MT performed worse than MT ($P < 0.01$) (Fig. 4F). Compared with the Control group, the Model group showed reduced time in the target quadrant, fewer quadrant entries, and fewer platform crossings

(all $P < 0.01$). MT significantly improved all three measures ($P < 0.01$). Relative to the Model group, ABX + MT did not significantly improve quadrant time ($P > 0.05$) but increased quadrant entries ($P < 0.05$) and platform crossings ($P < 0.01$). Compared with MT, ABX + MT showed reduced quadrant time ($P < 0.01$) and fewer quadrant entries ($P < 0.05$), with no difference in platform crossings ($P > 0.05$) (Fig. 4G-I). Representative heatmaps are shown in Fig. 4K.

In the nest-building test (48 h), Model group scores were significantly lower than Control ($P < 0.05$). MT significantly improved these scores ($P < 0.05$), while ABX + MT showed no significant difference from either Model ($P > 0.05$) or MT ($P > 0.05$) (Fig. 4J, L). Overall, ABX + MT attenuated most benefits of MT, indicating that MT alleviates cognitive dysfunction in AD mice partly via the gut microbiota. Specifically, ABX-induced microbiota depletion partially counteracted MT's effects on spatial memory and nest-building ability but had minimal impact on working memory and depression-like behaviors.

To further assess gut microbiota changes after ABX intervention in AD model mice, fecal samples were collected following the first week of ABX gavage for 16S rRNA sequencing. The Venn diagram showed 564 ASVs in the Model group and 289 in the ABX_Model group (Fig. 4M). PLS-DA revealed complete separation between the two groups, indicating distinct microbiota structures (Fig. 4N). Diversity indices (ACE, Chao1, Shannon, Simpson) were significantly lower in the ABX_Model group than in the Model group (all $P < 0.01$) (Fig. 4O). LEfSe identified core taxa (Fig. 4P). At the phylum level, the Model group contained 7.024% *Proteobacteria*, 52.080% *Bacteroidota*, 34.930% *Firmicutes*, 3.056% *Desulfobacterota*, and 2.092% *Campylobacterota*. In contrast, the ABX_Model group had 97.371%, 1.366%, 1.104%, 0.023%, and 0.005%, respectively (Fig. 4Q). Compared with the Model group, the ABX_Model group showed a significant increase in *Proteobacteria* ($P < 0.01$) and significant decreases in the other four phyla ($P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.05$), indicating that ABX treatment made *Proteobacteria* the dominant phylum while depleting other microbial groups.

Through experiments involving ABX-mediated interventions in AD model mice, it was determined that ABX intervention results in a significantly reduced gut microbiota load in these mice, and that the structure of this reduced gut microbiota exacerbates cognitive impairment in the AD model mice, revealing a close association between AD-related cognitive impairment and the gut microbiota.

3.5 MT improves intestinal-brain barrier integrity and monocarboxylic acid transporter expression levels in AD model mice, and this effect is influenced by the gut microbiota

In the microbiota-gut-brain axis, barrier function is crucial for metabolite exchange. This study assessed intestinal barrier and blood-brain barrier (BBB) integrity by quantifying MUC-2 and tight junction proteins (ZO-1, Occludin, Claudin-5) in colonic and brain tissues via immunohistochemistry.

In colonic tissue, compared with the Control group, the Model group showed significantly reduced ZO-1, Occludin, and MUC-2 expression ($P < 0.05$, $P < 0.05$, $P < 0.01$). MT intervention reversed these reductions (all $P < 0.01$). The ABX + MT group only upregulated Occludin ($P < 0.05$), and all three proteins were significantly lower than in the MT group (all $P < 0.01$) (Fig. 5A-D).

In brain tissue, the Model group exhibited reduced Occludin, ZO-1, and Claudin-5 expression (all $P < 0.01$). MT reversed all three (all $P < 0.01$). ABX + MT increased ZO-1 and Claudin-5 (both $P < 0.01$) but not Occludin ($P > 0.05$). Compared with MT, ABX + MT showed lower Occludin and Claudin-5 (both $P < 0.01$) but higher ZO-1 ($P < 0.01$) (Fig. 5E-H).

MCT-1, a key transporter for lactate, pyruvate, and SCFAs [27], was assessed in colon and brain tissues via immunofluorescence and WB. Compared with the Control group, MCT-1 expression was significantly reduced in both tissues in the Model group ($P < 0.05$ for colon, $P < 0.01$ for brain). MT intervention significantly upregulated MCT-1 in both tissues ($P < 0.05$, $P < 0.01$). In contrast, ABX + MT reduced colonic MCT-1 expression ($P < 0.05$) but had no significant effect on brain MCT-1 ($P > 0.05$). Moreover, MCT-1 levels in both tissues were significantly lower in the ABX + MT group than in the MT group (both $P < 0.01$) (Fig. 5I-K). These findings suggest that MT improves gut-brain barrier integrity and regulates monocarboxylate transporter expression in AD model mice, and these effects are modulated by the gut microbiota, indicating that the microbiota serves as a key mediator of MT's action on the gut-brain axis.

3.6 Mechanisms by which MT regulates intestinal-brain oxidative stress, peripheral iron overload, and central ferroptosis in AD model mice

The effects of MT on oxidative stress markers (CAT, GSH, T-SOD, and MDA) are shown in Figs. 6A-D. Compared with the Control group, the Model group exhibited significantly reduced levels of CAT, GSH, and T-SOD in brain tissue ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively) and elevated MDA levels ($P < 0.01$). MT intervention significantly reversed these changes, upregulating CAT, GSH, and T-SOD ($P < 0.01$, $P < 0.01$, $P < 0.05$) and reducing MDA ($P < 0.01$) compared with the Model group.

The effects of MT on colonic antioxidant capacity are shown in Figs. 6E-H. In the Model group, colonic CAT, GSH, and T-SOD levels were significantly lower than those in the Control group ($P < 0.05$, $P < 0.01$, $P < 0.05$), while MDA levels were elevated ($P < 0.01$). MT intervention significantly increased colonic CAT and T-SOD levels (both $P < 0.01$) and decreased MDA levels ($P < 0.01$) compared with the Model group; GSH showed an increasing trend but did not reach statistical significance ($P > 0.05$). Collectively, these results indicate that MT effectively enhances the antioxidant capacity of both brain and colon tissues in AD model mice.

Mechanistically, iron overload is one of the key factors contributing to oxidative stress and ferroptosis-induced damage. The Keap1/Nrf-2/HO-1 pathway serves as a critical defense system against oxidative stress. Western blot analysis revealed that, compared with the Control group, Keap-1 expression was significantly upregulated ($P < 0.01$) in brain tissue from the Model group, while Nrf-2 and HO-1 expression were downregulated ($P < 0.05$, $P < 0.01$). MT intervention reduced Keap-1 expression levels in the Model group ($P < 0.01$) and significantly increased Nrf-2 and HO-1 expression levels ($P < 0.01$, $P < 0.05$) (Fig. 6I-L). Furthermore, we conducted tissue iron content assays, which revealed that iron levels in the plasma and colon tissues of mice in the Model group were significantly higher than those in the Control group ($P < 0.01$ for both), indicating iron overload. MT intervention significantly reversed iron

overload in the plasma and colon tissues of AD model mice ($P < 0.05$, $P < 0.01$) (Fig. 6M-N). Iron overload, a hallmark of iron metabolism dysregulation, is a key factor in inducing ferroptosis.

To elucidate the mechanism by which MT alleviates the pathological progression of AD through the regulation of ferroptosis, we focused on the proteins IREB2, DJ-1, Gpx-4, and FTH, which maintain intracellular iron homeostasis and regulate ferroptosis. The results showed that, compared with the Control group, the expression levels of Gpx-4 and DJ-1 in the brains of mice in the Model group were significantly reduced ($P < 0.01$ for both). MT intervention significantly increased the expression levels of Gpx-4 and DJ-1 in the brains of AD model mice ($P < 0.01$ for both) (Fig. 6O-Q). WB analysis further supported these findings regarding Gpx-4 and DJ-1. Furthermore, compared with the Control group, IREB2 expression was significantly upregulated ($P < 0.01$) and FTH expression was downregulated ($P < 0.01$) in the Model group, whereas MT intervention reduced IREB2 expression ($P < 0.01$) and upregulated FTH expression ($P < 0.01$) in the brains of model mice (Fig. 6R-V). These data indicate that MT effectively enhances the antioxidant capacity of brain and colon tissues in AD model mice, alleviates iron overload, upregulates DJ-1, Gpx-4, and FTH expression, downregulates IREB2 expression, and modulates the Keap-1/Nrf-2/HO-1 signaling pathway, thereby inhibiting ferroptosis in central neurons and exerting a neuroprotective effect.

3.7 FMT using feces from MT-treated mice improves cognitive dysfunction and alters gut microbiome composition similarly to MT in AD model mice

To verify whether MT-remodeled gut microbiota is key to neuroprotection, FMT (FMT_MT) was performed in AD model mice (Fig. 7A). Body weight in the Model group remained consistently lower than in the Control group; FMT_MT showed a similar trend to Control after week 4, with no significant difference from Model ($P > 0.05$) (Fig. 7B). Behaviorally, FMT_MT did not significantly increase spontaneous alternation in the Y-maze ($P > 0.05$) but significantly reduced immobility time in the tail suspension test ($P < 0.01$) (Fig. 7C-D). In the Morris water maze, by Day 3, FMT_MT significantly shortened escape latency versus Model ($P < 0.01$) (Fig. 7E). In the spatial exploration test, Model showed reduced target quadrant time and fewer platform crossings ($P < 0.05$, $P < 0.01$), while FMT_MT significantly improved both (both $P < 0.05$) (Fig. 7F-G, K). In the nest-building test, Model scores were lower than Control from 12 h onward ($P < 0.01$); FMT_MT produced more complete nests and significantly higher scores at 48 h ($P < 0.01$) (Fig. 7H-I).

To rule out the possibility that the effects of FMT_MT intervention were due to residual MT in the donor feces, HPLC was used to detect residual MT in mouse feces and brain tissue 24 hours after MT gavage. As shown in Fig. 8K, the retention time of the MT standard at a detection wavelength of 222 nm was 4.37 min. An MT chromatographic peak was also observed at 4.37 min in the brain tissue samples, indicating that MT can enter the brain tissue after oral administration. However, no characteristic MT peak was observed at the same retention time in the fecal samples, suggesting that there were no or only extremely low levels of MT residues in the donor feces (Fig. 7J).

16S rRNA sequencing analysis was performed following a 6-week FMT_MT intervention. Analysis of ASVs clustered at 100% sequence similarity revealed that 344 ASVs were detected in the Model group and 360 ASVs in the FMT_MT group (Fig. 7L). Compared with the Model group, the ACE, Chao1, Simpson, and Shannon indices were all significantly lower in the FMT_MT group ($P < 0.01$, $P < 0.01$, $P > 0.05$, $P < 0.05$) (Fig. 7P), indicating that the FMT_MT intervention reduced gut microbiota diversity in model mice. PCoA analysis results showed a large distance between the FMT_MT intervention group and the Model group, with clear differentiation, confirming significant differences in the species composition of the gut microbiota between the two groups (Fig. 7M). At the phylum level, the gut microbial composition of mice in the Model and FMT_MT groups primarily consisted of *Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, *Actinobacteriota*, and *Proteobacteria*. Specifically, the proportions of these phyla in the Model group were 83.892%, 10.841%, 0.003%, 3.099%, and 0.499%, respectively, while those in the FMT_MT group were 59.579%, 23.063%, 7.475%, 3.225%, and 3.835%, respectively. In contrast, the proportion of *Firmicutes* in the gut of mice in the FMT_MT group was lower than that in the Model group, while the proportions of *Bacteroidota*, *Verrucomicrobiota*, *Actinobacteriota*, and *Proteobacteria* were relatively higher (Fig. 7O).

The *Firmicutes/Bacteroidota* (F/B) ratio is crucial for maintaining intestinal homeostasis, and its elevation is closely associated with increased inflammation. Notably, compared with the Model group, the F/B ratio was significantly lower in the FMT_MT group ($P < 0.01$) (Fig. 7N). Further analysis of the differential microbiota across groups revealed that, compared to the Model group, the FMT_MT group also exhibited increased *Bacteroidota* abundance at the phylum level ($P > 0.05$), which is consistent with the trend of *Bacteroidota* changes induced by MT intervention. At the family level, the FMT_MT group significantly reversed the abnormal increases in *Acidaminococcaceae* ($P < 0.05$), *Erysipelotrichaceae* ($P < 0.01$), and *Oscillospiraceae* ($P < 0.01$). At the genus level, the abundances of *Anaerovibrio*, *Dialister*, *Fournierella*, *Lachnospira*, and *Olsenella* were all significantly suppressed in the FMT_MT group ($P < 0.01$ for all), which was largely consistent with the downregulated trends of *Dialister*, *Fournierella*, *Lachnospira*, and *Olsenella* in the MT group (Fig. 7Q). These analyses confirm that FMT_MT can mimic the gut microbiota restructuring effects of MT on AD model mice, further indicating that the reconfigured gut microbiota structure is a key factor in the neuroprotective effects of MT.

3.8 The gut microbiota reshaped by MT mediates MT's regulatory role in ferroptosis in AD

Through FMT experiments, we further investigated whether the gut microbiota reshaped by MT mediates MT's regulatory effect on ferroptosis in AD. WB results showed that, compared to the Model group, the FMT_MT group significantly reduced the markedly elevated levels of phosphorylated Tau and $A\beta_{1-42}$ in the brains of AD model mice ($P < 0.01$ for both) and significantly increased NeuN protein expression levels ($P < 0.01$) (Fig. 8A-D). Immunofluorescence staining further confirmed these findings (Fig. 8E-G), demonstrating that the FMT_MT intervention significantly suppressed $A\beta$ levels in the brains of AD model mice while significantly enhancing NeuN protein levels ($P < 0.01$ for both).

At the same time, we confirmed that FMT_MT intervention modulates the Keap-1/Nrf-2/HO-1 pathway to mediate antioxidant defense mechanisms. Specifically, compared with the Control group, Keap-1 expression levels in the brains of mice in the Model group were significantly elevated ($P < 0.05$), while Nrf-2 and HO-1 expression levels were significantly reduced ($P < 0.01$, $P < 0.05$). The FMT_MT intervention significantly reduced Keap-1 expression levels in the brains of AD model mice ($P < 0.05$) and significantly upregulated Nrf-2 and HO-1 expression levels ($P < 0.05$, $P < 0.01$) (Fig. 8H-K). Furthermore, examination of the expression levels of proteins involved in ferroptosis-related pathways revealed that IREB2 expression was significantly higher in the Model group than in the Control group ($P < 0.05$), while DJ-1 and FTH expression levels were significantly reduced ($P < 0.05$, $P < 0.01$). Following FMT_MT intervention, the elevated IREB-2 expression in the brains of AD model mice was reversed ($P < 0.05$), and the expression levels of DJ-1 and FTH proteins were significantly increased ($P < 0.01$ for both) (Fig. 8L-O). Immunofluorescence staining further confirmed these findings: compared with the Control group, the expression levels of DJ-1 and Gpx-4 in the brains of the Model group were significantly reduced ($P < 0.01$ for both), whereas in the FMT_MT group, the expression of both DJ-1 and Gpx-4 was significantly restored ($P < 0.01$ for both) (Fig. 8P-R). These results are consistent with the previously reported findings that MT modulates the ferroptosis pathway in AD. Thus, we have demonstrated that MT mediates its beneficial effects on AD cognitive impairment and pathology by reshaping the gut microbiota and have mechanistically elucidated its regulatory role in the ferroptosis pathway.

Discussion

The ICR mice used in this study are a strain that has been extensively employed in AD research, with well-established applications in multiple AD-like models, including A β toxicity, neuroinflammation, and oxidative stress [28, 29]. Aluminum is a known neurotoxic element implicated in the pathogenesis of Alzheimer's disease (AD)[30]. Previous studies have demonstrated that long-term low-dose D-galactose (D-gal) and aluminum chloride (AlCl₃) induction can mimic the natural aging process in mice, with pathological features including cholinergic dysfunction, cognitive decline, oxidative stress, accumulation of advanced glycation end products (AGEs), and biochemical and morphological changes in astrocytes [31]. The combination of D-gal and AlCl₃ has been widely validated to cause cognitive impairment and hippocampal pyramidal cell degeneration in mice. In this study, AD model mice generated by combined D-gal and AlCl₃ induction exhibited significant declines in learning and memory abilities, as well as AD-like pathological features and damage.

MT is an endogenous indoleamine compound. Tan et al. first reported in 1993 that MT possesses potent antioxidant activity [32]. Extensive evidence indicates that endogenous MT levels in the body decline with aging [33]. Zhou et al. reported a more pronounced decrease in MT levels in the cerebrospinal fluid of AD patients [34]. Multiple studies have revealed the multifaceted neuroprotective potential of MT. Regarding the prevention of AD pathology, Corpas et al. reported that MT reversed dementia-related anxiety and apathy in 3xTg-AD mice and reduced A β and P-Tau protein burdens [35]. Chang et al. further noted that in sleep-deprivation-induced cognitive abnormalities and neurological dysfunction, MT exerts its effects by promoting p-Nrf2 expression and the activity of downstream antioxidant enzymes (SOD,

CAT, GSH-Px) [36]. Furthermore, recent studies have revealed that in a sodium sulfite-induced osteoporotic mouse model, MT upregulates the expression of ferroptosis regulators Slc7A11, FTH1, and Gpx-4, thereby alleviating oxidative stress and mitigating bone loss [37].

MT demonstrated a synergistic alleviating effect on gut-brain damage in AD. We found that MT effectively alleviated cognitive and behavioral abnormalities and hippocampal neuronal damage in AD model mice, reduced intracranial A β levels and tau protein hyperphosphorylation, increased intracranial antioxidant enzyme activity, and inhibited excessive microglial activation. Additionally, we observed significant restoration of colon length and improvement in colon tissue morphology in AD model mice treated with MT, confirming that MT also effectively alleviates peripheral intestinal pathological dysfunction in AD. Furthermore, both brain and colon tissues in AD model mice exhibited consistent oxidative stress imbalances, characterized by significantly reduced SOD and CAT activity, significantly decreased GSH levels, and significantly elevated MDA levels. In mice treated with MT, these oxidative stress markers in both brain and colon tissues were significantly restored.

Regarding two critical structural defense systems in the body—the intestinal barrier and the blood-brain barrier—multiple studies have shown that AD mice often exhibit impaired intestinal barrier function, manifesting pathological phenomena such as colitis and altered intestinal permeability [38, 39]. The blood-brain barrier (BBB) serves as a key gateway controlling the movement of molecules and nutrients in and out of the brain, with its primary role being to maintain the homeostasis of the central nervous system [40, 41]. Compromised barrier integrity in AD development is primarily manifested by downregulated expression of tight junction proteins (TJs) and impaired intestinal mucosal barrier function [42]. Mucins (such as MUC-2), as major components of the intestinal mucus layer, can enhance the chemical defense of the intestinal barrier [43]. In this study, AD model mice exhibited downregulated expression of key TJs and MUC2 in the gut-brain barrier, resulting in impaired intestinal barrier and BBB integrity. MT intervention increased the expression of ZO-1 and Occludin in colonic TJs, promoted the secretion of the mucin MUC-2, and enhanced the expression of ZO-1, Occludin, and Claudin-5 in brain tissue. This intervention restored the integrity of the intestinal and blood-brain barriers in AD model mice and effectively reduced abnormal permeability in both the intestinal and blood-brain barriers. Additionally, the expression of small-molecule transporters influences the systemic distribution of metabolites. Monocarboxylic acid transporter 1 (MCT-1) is a key transporter for the transmembrane transport of lactate, ketone bodies, and SCFAs, playing a central role in substance exchange between the intestinal epithelium and the central nervous system. In this study, MT intervention not only increased the abundance of SCFA-producing bacteria but also elevated MCT-1 expression levels in the colon and brain tissues of AD model mice, thereby regulating SCFA transport and distribution. SCFAs enhance intestinal barrier integrity and help maintain host homeostasis [44], playing a key role in barrier protection. However, ABX combined with MT intervention completely suppressed the protective effect of MT on gut-brain barrier integrity, indicating that the gut microbiota influences the exertion of MT's effects.

Interventional drugs administered via the gastrointestinal tract interact with the gut microbiota to some extent. The gut microbiota serves as a complex and critical hub for bidirectional communication within

the gut-brain axis. Gut microbes are involved in numerous key physiological processes in the host, such as carbohydrate breakdown, SCFA production, immune development, and regulation of intestinal motility [45]. Numerous studies have demonstrated that dysbiosis of the gut microbiota is closely associated with the pathogenesis of various central nervous system disorders, including AD; for instance, the neuroinflammation it induces is considered one of the core drivers of AD progression. Based on these considerations, we progressively conducted experiments related to the gut microbiota. We used an ABX therapy to induce a low gut microbiota burden in mice. Not only did we confirm that ABX pretreatment significantly reduced gut microbiota diversity and abundance in AD model mice, but we also found that ABX treatment exacerbated cognitive impairment in AD mice. This is consistent with the findings reported by Mosaferi et al., who demonstrated that antibiotic-induced reduction in gut microbiota exacerbates spatial memory impairment in AD model mice, with marked cognitive decline observed in both Y-maze and water maze tests [46]. Further studies found that following combined ABX and MT intervention, the neuroprotective effects of MT on spatial memory, nest-building ability, P-Tau and NeuN expression, and microglial activation in AD model mice were significantly attenuated, indicating that the neuroprotective effects of MT are partially dependent on the structure of the gut microbiota. Edward et al. also confirmed that antibiotic-induced gut dysbiosis increased A β and P-Tau expression in the hippocampus of 3xTg-AD mice, suggesting that the gut microbiome profile is a key factor influencing the progression of AD pathology [47].

Consequently, we examined the gut microbiota of AD model mice following MT intervention and found that MT intervention effectively reshaped the gut microbiota structure of these mice, as evidenced by an increase in the relative abundance of SCFA-producing Bacteroidota at the phylum level. *Bacteroidota* is a key microbial group in the human gut. Shin et al. noted that *Bacteroidota* is a major contributor to maintaining the symbiotic homeostasis of the human gastrointestinal tract, and that SCFAs produced by *Bacteroidota*, such as acetate, butyrate, and propionate, serve as primary end products that exert broad and beneficial effects on the host [48]. At other taxonomic levels, the abundances of *o_Erysipelotrichales*, *f_Erysipelotrichaceae*, *f_Oscillospiraceae*, *g_Fournierella*, *g_Lachnospira*, *g_Olsenella*, *g_Turicibacter*, and *g_Dialister* were suppressed by MT intervention. Zheng Zhu et al. found that the abundance of Erysipelotrichaceae increased as cognitive status shifted toward AD [49]. Mena-Vázquez et al. found that *Dialister* was significantly enriched in the intestines of obese RA patients, and its abundance was positively correlated with markers of obesity-related inflammation. Liu et al. reported that *Turicibacter* abundance was elevated in a colitis model of aging mice, consistent with intestinal barrier damage and exacerbated inflammation. Liu et al. confirmed in patients with chronic constipation that *Oscillospiraceae* abundance was positively correlated with levels of pro-inflammatory metabolites [50–52]. Collectively, these results suggest that MT may exert its neuroprotective effects by upregulating beneficial bacteria that produce SCFAs and suppressing key harmful bacterial genera. This finding was further corroborated in ABX-treated AD mice: the relative abundance of *Proteobacteria* in the feces of ABX-treated AD mice rose sharply, while commensal bacteria such as *Bacteroidota* and *Firmicutes* were severely depleted; this group of mice exhibited worsening cognitive impairment in behavioral tests.

To verify whether MT's effects on improving AD pathology and cognitive impairment are related to the gut microbiota, this study further conducted a fecal microbiota transplantation (FMT) experiment in mice from the MT intervention group (FMT_MT group). Prior to the experiment, we first ruled out the possibility that residual MT in donor feces might directly interfere with the recipient mice. HPLC results showed that chromatographic peaks consistent with the retention time of the MT standard were detectable in brain tissue samples, indicating that orally administered MT can enter brain tissue; however, no characteristic MT peaks were found in fecal samples, preliminarily suggesting that there were no or extremely low levels of MT residue in donor feces. This ruled out the direct interfering effect of MT in the FMT_MT intervention. Validation experiments showed that the gut microbiota structure of AD model mice treated with fecal microbiota transplantation (FMT_MT) was highly similar to that of the MT group. Specifically, the F/B ratio decreased, and the abundance of *Bacteroidota* showed an upward trend; simultaneously, the abundance of genera such as *f_Erysipelotrichaceae*, *f_Oscillospiraceae*, *g_Dialister*, *g_Fournierella*, *g_Lachnospira*, and *g_Olsenella* was suppressed. At the phenotypic level, FMT_MT not only significantly improved spatial learning and memory abilities and reduced depression-like behaviors in AD model mice but also decreased the expression levels of pathological proteins A β and P-Tau and significantly enhanced the expression of the neuronal marker NeuN. These neuroprotective effects, both behavioral and pathological, were highly consistent with the outcomes of direct MT administration. These results establish the causal relationship underlying the hypothesis that "gut microbiota mediate the anti-AD effects of MT via the gut-brain axis." However, it should be noted that the mediating role of gut microbiota may only account for part of the mechanism underlying MT's anti-AD effects; MT may also cross the blood-brain barrier after oral administration and exert direct neuroprotective effects.

In this study, iron levels in the colon and blood of AD model mice were significantly elevated, suggesting the presence of intestinal iron overload in the AD model, which may lead to abnormal iron metabolism in the brain via the circulatory system. MT intervention effectively reversed iron overload in the plasma and colon tissue of AD model mice. Iron is essential for maintaining the body's metabolism. However, iron overload promotes the production of reactive oxygen species (ROS) and causes damage to cells and tissues, making it a key factor in exacerbating oxidative stress and ferroptosis-related damage. Intestinal iron overload can disrupt the iron homeostasis of the intestinal microenvironment, thereby reshaping the gut microbiota and compromising the integrity of the intestinal barrier [53]. When free iron ions accumulate abnormally in the brain, they can drive ferroptosis by inducing excessive ROS production. In this study, MT intervention effectively enhanced the antioxidant capacity of brain and colon tissues in AD model mice, significantly upregulating CAT and T-SOD levels while reducing MDA levels. Wang et al. proposed that A β and hyperphosphorylated tau can exacerbate iron deposition in the brain, creating a vicious cycle between AD progression and iron deposition [54]. Therefore, modulating the ferroptosis pathway holds potential for delaying the pathological progression of AD.

The Keap-1/Nrf-2/HO-1 pathway is a critical defense system against oxidative stress. When oxidative stress occurs, Keap-1 undergoes a conformational change, which inhibits the degradation of Nrf-2, leading to its accumulation and translocation into the nucleus, where it activates downstream

antioxidant genes such as HO-1 and anti-inflammatory mediators. Studies have shown that DJ-1 can dissociate Nrf-2, allowing it to translocate to the nucleus and activate the expression of antioxidant enzymes, including Gpx-4 and SOD [55]. Among these, Gpx-4 is a key effector protein that inhibits lipid peroxidation and ferroptosis. It uses the thiol group of GSH as an electron donor; depletion of GSH leads to the inactivation of Gpx-4, resulting in the accumulation of lipid peroxides and the induction of ferroptosis [56]. Iron regulatory protein IREB2, as a central mediator of intracellular iron homeostasis, collaboratively regulates iron metabolism by binding to iron-responsive elements (IREs) on the transcripts of ferritin (FTH/FTL) and other iron metabolism-related genes. Elevated IREB2 protein levels disrupt iron homeostasis, leading to the accumulation of the labile iron pool (LIP) and exacerbating lipid peroxidation [57]. In this study, MT intervention activated the Keap-1/Nrf-2/HO-1 signaling pathway, a mechanism likely associated with the upregulation of FTH, DJ-1, and Gpx-4, as well as the downregulation of IREB2 expression. Interestingly, FMT_MT intervention similarly activated the Keap-1/Nrf-2/HO-1 signaling pathway and synchronously regulated the expression levels of the aforementioned iron metabolism-related proteins, further confirming that MT mediates the gut-brain antioxidant defense system by reshaping the gut microbiota structure and metabolism, thereby inhibiting neuronal ferroptosis.

In summary, this study reveals the core mechanism by which the gut microbiota mediates MT-induced suppression of ferroptosis and exerts neuroprotective effects in AD model mice. Specifically, MT suppresses the expression of AD pathological proteins, alleviates oxidative stress, and restores intestinal barrier and BBB integrity by reshaping the gut microbiota structure and metabolism—a process partially mediated by the gut microbiota—thereby inhibiting neuronal ferroptosis and ultimately improving AD pathology and cognitive impairment (Fig. 9). This study closely links the gut microbiota with the ferroptosis pathway, providing important experimental support for the role of the gut microbiota in mediating MT's suppression of ferroptosis. This study has certain limitations: first, there is a lack of mechanistic validation at the cellular level; second, no repopulation experiments were conducted to validate the identified key bacterial strains; furthermore, whether MT exerts part of its effect in improving AD pathology through direct entry into the brain remains to be further investigated. Nevertheless, this study offers a new perspective for gut microbiota-mediated drug interventions in the prevention and treatment of AD.

Abbreviations

AD
Alzheimer's disease
MT
melatonin
AlCl₃
aluminum trichloride
D-gal
D-galactose

A β
 β -amyloid
P-Tau
phosphorylated tau protein
CNS
central nervous system
HE
hematoxylin-eosin staining
ABX
antibiotics
FMT
fecal microbiota transplantation
SCFAs
short-chain fatty acids
NeuN
neuronal nuclei
CAT
catalase
MDA
malondialdehyde
GSH
reduced glutathione
T-SOD
total superoxide dismutase
GAPDH
glyceraldehyde-3-phosphate dehydrogenase
IBA-1
ionized calcium-binding adaptor molecule 1
ZO-1
zonulin-1
MUC-2
mucin 2
Claudin-5
tight junction protein 5
MCT-1
monocarboxylate transporter 1
Keap-1
Kelch-like ECH-related protein 1
Nrf-2
nuclear factor erythropoietin-related factor 2

HO-1
heme oxygenase-1
IREB2
iron response element-binding protein 2
Gpx-4
glutathione peroxidase 4
DJ-1
protein encoded by the PARK7 gene
FTH
ferritin heavy chain 1
BBB
blood-brain barrier
TJs
tight junctions.

Declarations

Ethics approval

All experiments conducted at the institute were in strict compliance with the provisions of China's regulations on the management of experimental animals and were approved by the Ethical Review Committee for Experimental Animals of Xinhua Hospital Affiliated to Dalian University (Approval No.: k2024-002). Clinical trial number: not applicable.

Conflicts of interest

There are no conflicts to declare.

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Author Contribution

Dan Pu, Ju Wu and Xiaofei Han conceived the study. Dan Pu and Xiaofei Han designed the research; Dan Pu, Yanjie Dou, Wei Zhang and Yaohui Wang performed the experiments; Longxing Wang and Xiaofei Han contributed essential reagents or tools. Dan Pu analyzed the data. Dan Pu and Xiaofei Han wrote the paper. Ju Wu and Longxing Wang provided suggestions. All authors have read and approved the article.

Data availability

The data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1

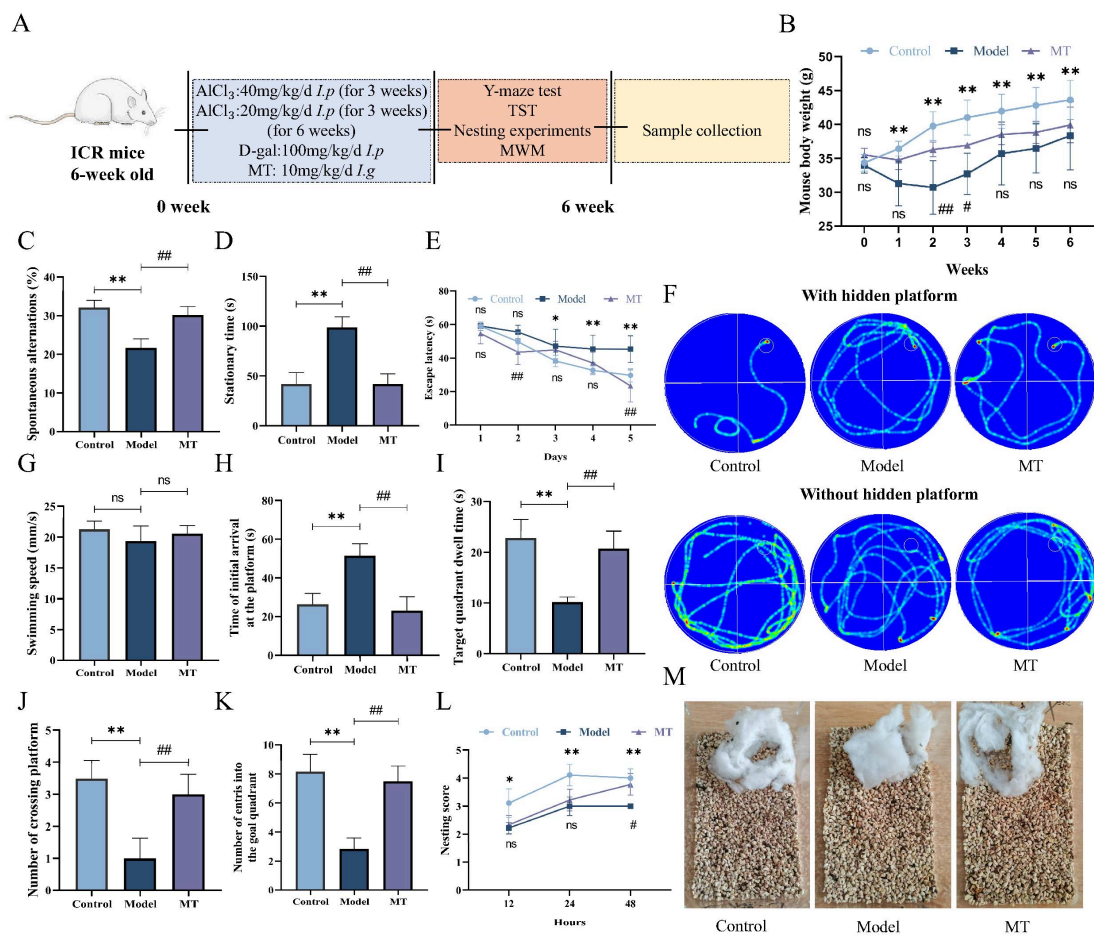


Figure 1

MT alleviates cognitive dysfunction in a mouse model of AD. (A) Schematic diagram of the experimental design. (B) Body weight of mice. (C) Spontaneous alternation rate in the Y-maze test. (D) Immobile time in the tail-suspension test. (E) Escape latency in the spatial navigation test. (F) Representative heatmap of swim tracks in the water maze test. (G) Mean swimming speed. (H) Time to first reach the platform. (I) Dwell time in the target quadrant. (J) Number of platform crossings. (K)

Number of entries into the target quadrant. (L) Nesting score in the nesting test. (M) Representative image from the nesting test. Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, and the # group with the Model group, respectively).

Figure 2

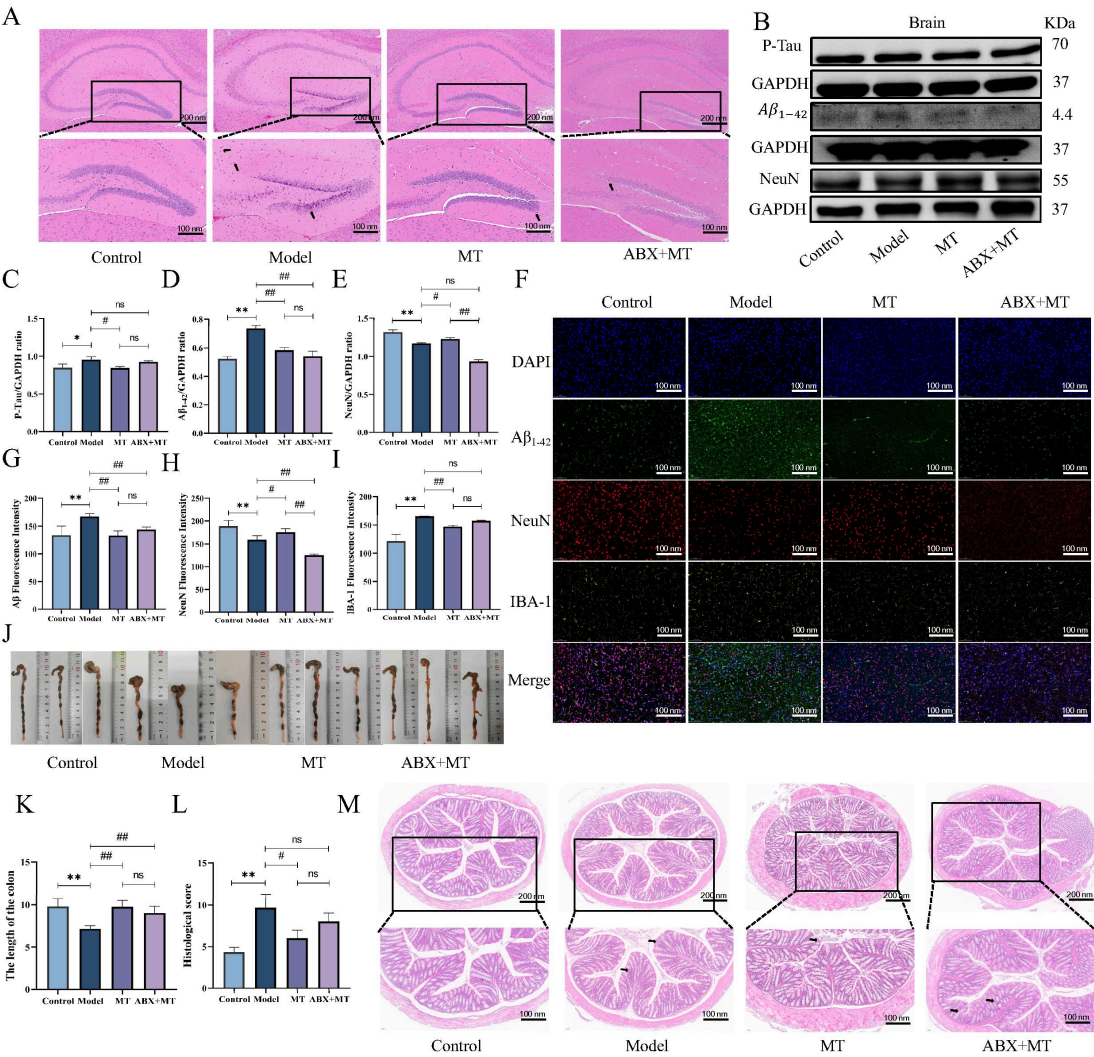


Figure 2

MT simultaneously alleviates neuronal damage and intestinal dysfunction in AD model mice. (A) Representative HE staining of brain tissue (scale bar: 200 μ m, 100 μ m). (B-E) Representative Western blot images and grayscale analysis of P-Tau, A β_{1-42} and NeuN in the brain (with GAPDH as the internal control). (F) Representative immunofluorescence images of A β , NeuN, and IBA-1 in the brain (scale bar: 100 μ m). (G) Quantitative analysis of fluorescence intensity for A β , (H) NeuN, and (I) IBA-1. (J) Representative photographs of the colon in each group. (K) Analysis of colon length. (L) Histological scoring analysis of the colon. (M) Representative HE staining results of colon tissue (scale bar: 200 μ m, 100 μ m). Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, the # group with the Model group, and the MT group, respectively).

Figure 3

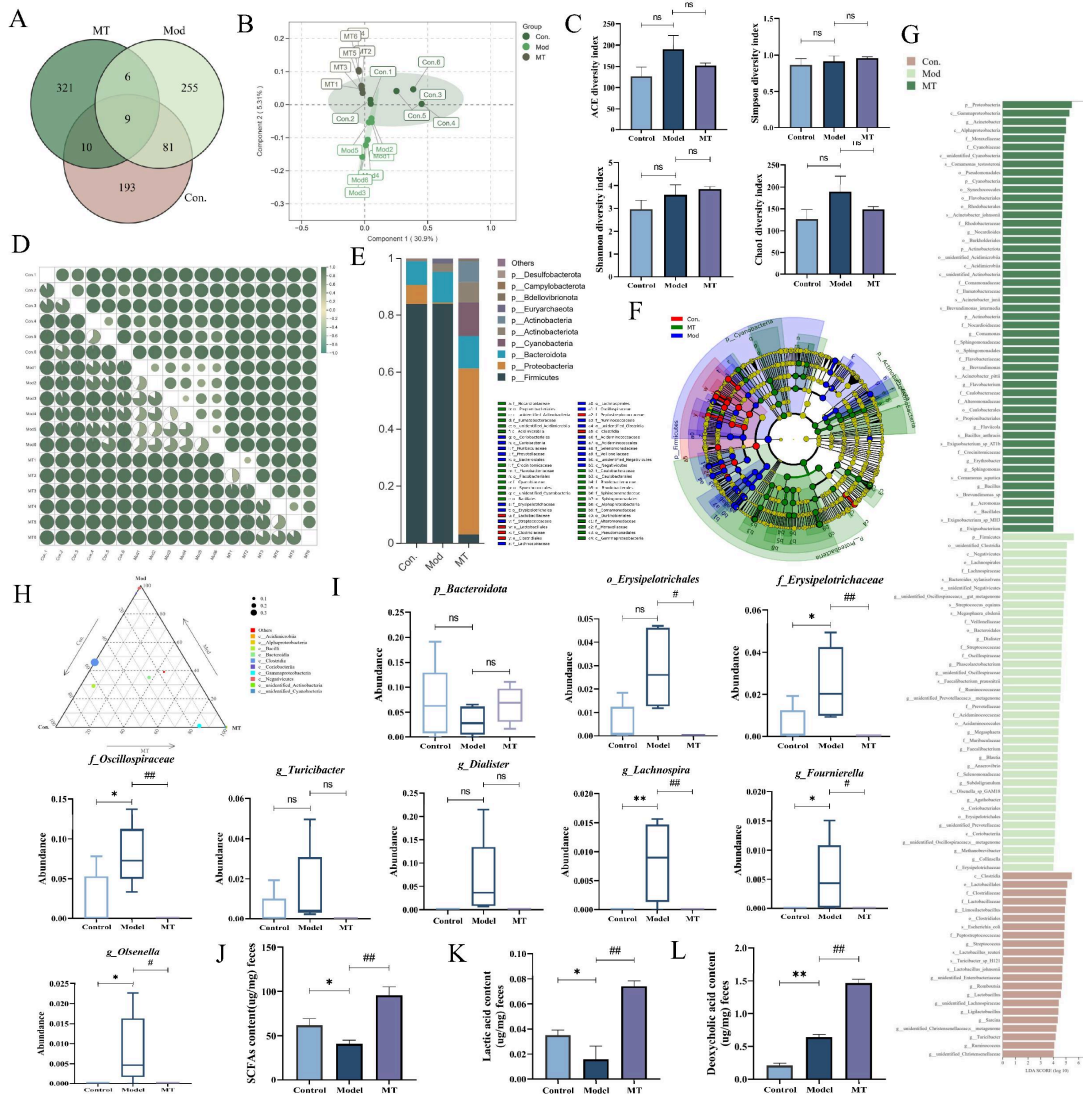


Figure 3

MT restores gut microbiota dysbiosis and microbial metabolism in AD model mice. (A) Venn diagram of shared and unique OTUs (operational taxonomic units) across groups. (B) PLS-DA plot. (C) ACE index, Chao-1 index, Shannon index, and Simpson index for evaluating gut microbial richness and evenness. (D) Heatmap of the distance matrix showing microbial community differences between groups. (E) Phylum-level composition and community abundance percentages in mice across groups. (F) LEfSe analysis to

identify taxonomic units with differential abundance between groups. (G) LDA score plot. (H) Triangular phase diagram at the class level. (I) Relative abundances of differentially abundant bacteria in the gut microbiota of each group. Fecal concentrations of (J) total SCFAs, (K) lactic acid, and (L) deoxycholic acid. Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group and the # group with the Model group, respectively).

Figure 4

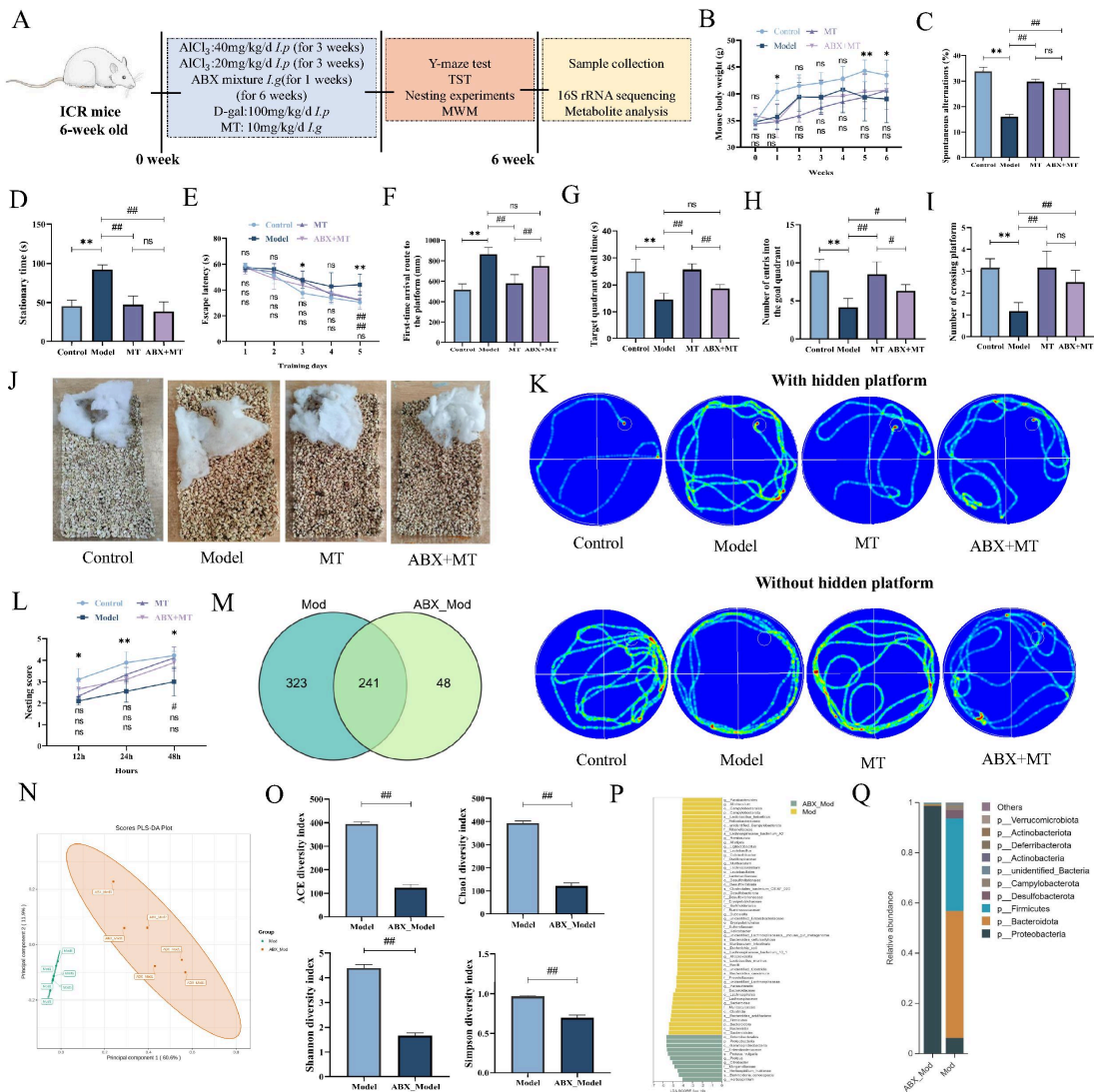


Figure 4

ABX attenuates the ameliorative effects of MT on cognitive dysfunction in AD model mice. (A)

Schematic diagram of the experimental design. (B) Mouse body weight. (C) Spontaneous alternation rate in the Y-maze test. (D) Immobile time in the tail-suspension test. (E) Escape latency in the spatial navigation test. (F) Distance to first platform reach. (G) Time spent in the target quadrant. (H) Number of entries into the target quadrant. (I) Number of platform crossings. (J) Nesting score in the nesting test. (K) Representative heatmap of trajectories in the water maze test. (L) Representative image from the nesting test. (M) Venn diagram of shared and unique OTUs (Operational Taxonomic Units) across groups. (N) PLS-DA plot. (O) ACE, Chao1, Shannon, and Simpson indices for assessing gut microbial richness and evenness. (P) LDA score plot. (Q) Phylum-level composition and community abundance percentages for each group. Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, the # group with the Model group, and the MT group, respectively).

Figure 5

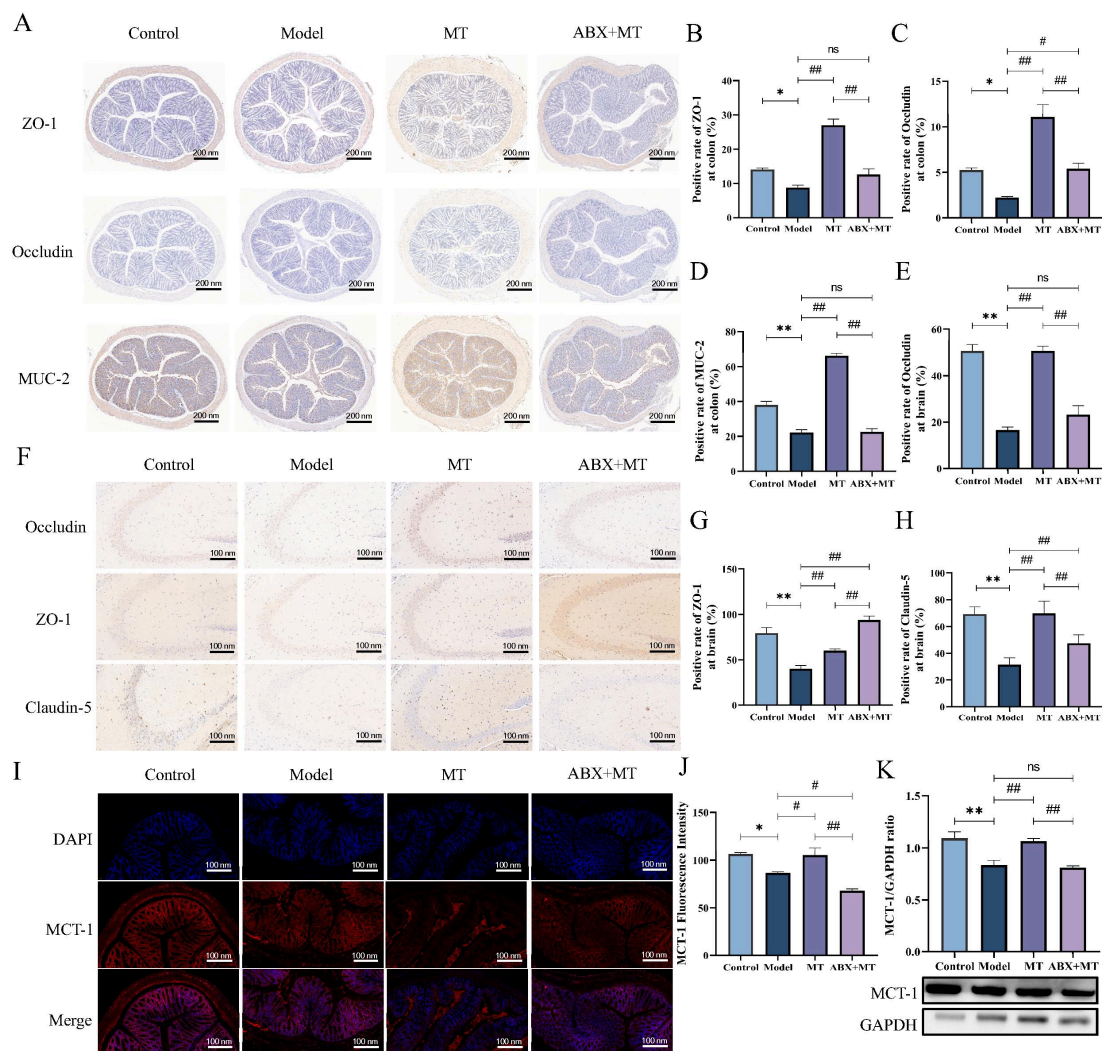


Figure 5

MT improves intestinal-brain barrier integrity and monocarboxylate transporter expression levels in AD model mice, and this effect is influenced by the gut microbiota. (A) Representative immunohistochemical images of ZO-1, Occludin, and MUC-2 in colonic tissue (scale bar: 200 μ m). (B) Percentage of ZO-1-positive cells in colonic tissue. (C) Quantitative analysis of Occludin protein expression in colon tissue. (D) Quantitative analysis of MUC-2 protein expression in colon tissue. (E)

Quantitative analysis of Occludin protein expression in brain tissue. (F) Representative immunohistochemical images of Occludin, ZO-1, and Claudin-5 in brain tissue (scale bar: 100 μ m). (G) Quantitative analysis of ZO-1 protein expression in brain tissue. (H) Quantitative analysis of Claudin-5 protein expression in brain tissue. (I) Representative immunofluorescence image of MCT-1 in colon tissue (scale bar: 100 μ m). (J) Quantitative analysis of MCT-1 fluorescence intensity. (K) Representative Western blot image of MCT-1 in brain tissue and analysis of gray-scale values (with GAPDH as the internal control). Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, the # group with the Model group, and the MT group, respectively).

Figure 6

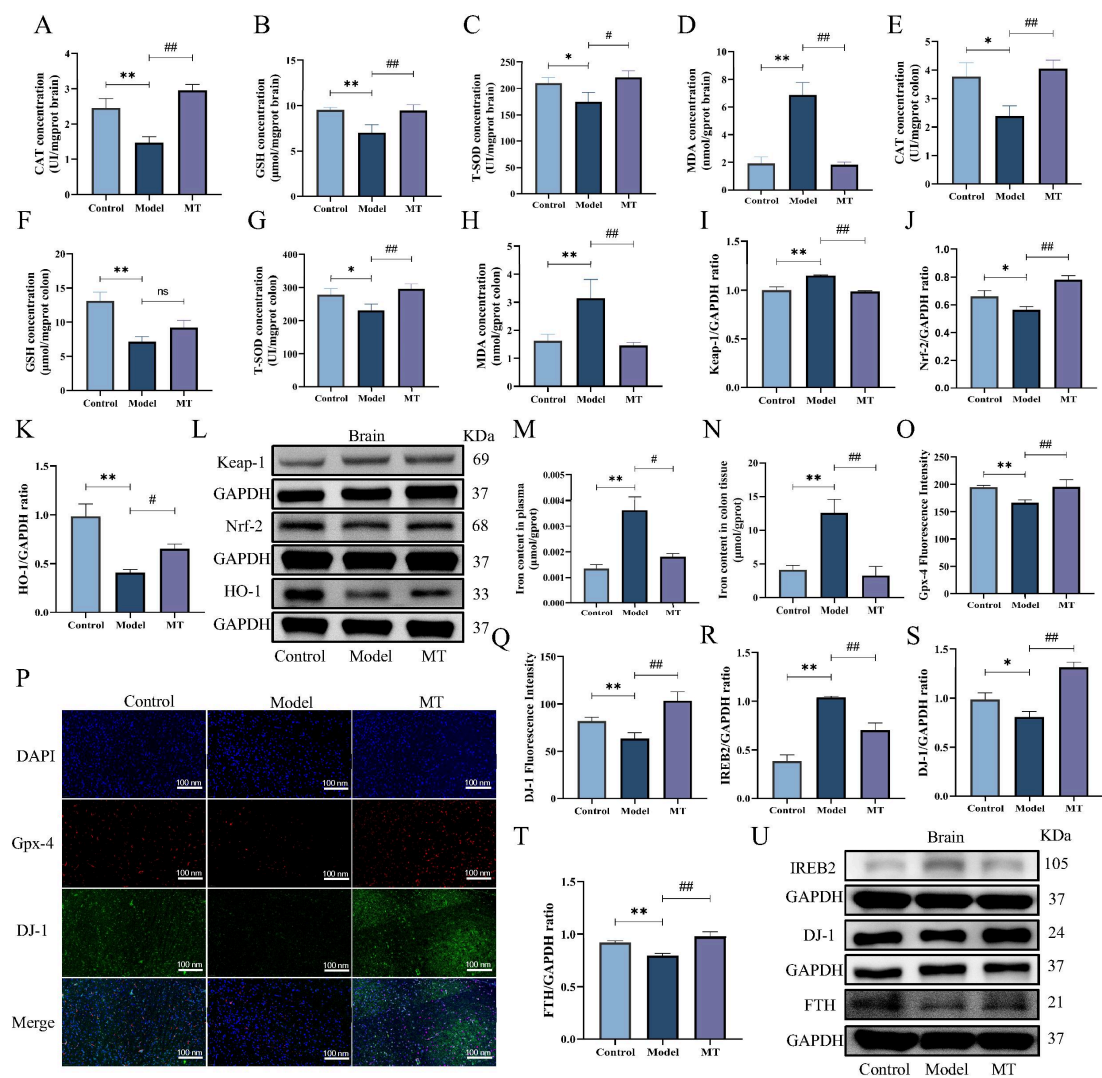


Figure 6

Mechanism of MT regulation of gut-brain oxidative stress, peripheral iron overload, and central ferroptosis in AD model mice. (A-D) Levels of CAT, GSH, T-SOD, and MDA in brain tissue. (E-H) Levels of CAT, GSH, T-SOD, and MDA in colon tissue. (I-L) Representative Western blot images and grayscale analysis of Keap-1, Nrf-2, and HO-1 in brain tissue (with GAPDH as an internal control). (M-N) Analysis of iron content in plasma and colon tissue. (O) Quantitative analysis of Gpx-4 fluorescence intensity. (P)

Representative immunofluorescence images of Gpx-4 and DJ-1 in brain tissue (scale bar: 100 μ m). (Q) Quantitative analysis of DJ-1 fluorescence intensity. (R-U) Representative Western blot images and grayscale value analysis of IREB2, DJ-1, and FTH in brain tissue (with GAPDH as the internal control). Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, and the # group with the Model group, respectively).

Figure 7

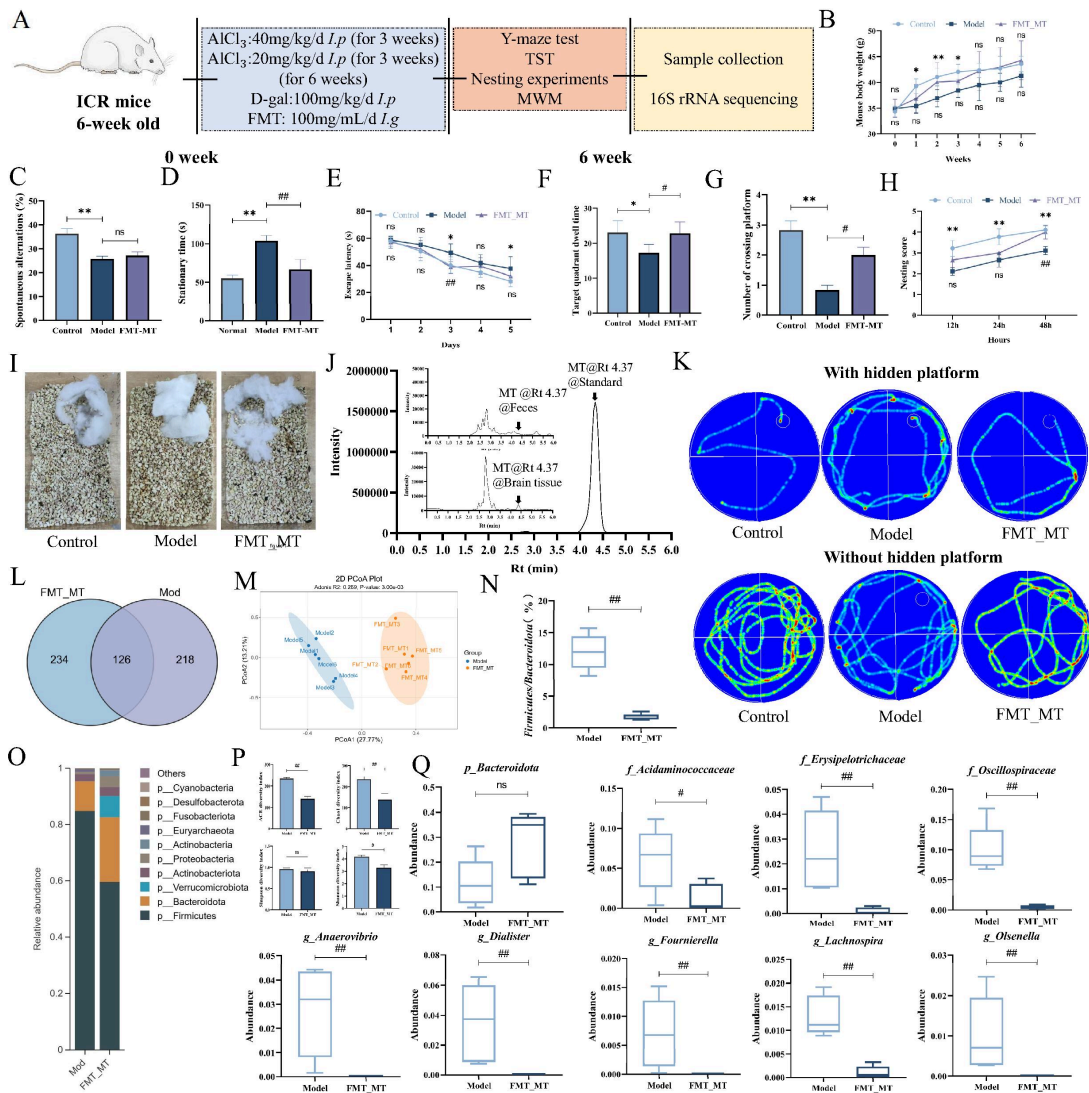


Figure 7

FMT using feces from mice treated with MT still improves cognitive dysfunction in AD model mice. (A) Schematic diagram of the experimental design. (B) Body weight of mice. (C) Spontaneous alternation rate in the Y-maze test. (D) Immobile time in the tail-suspension test. (E) Escape latency in the spatial navigation test. (F) Dwell time in the target quadrant. (G) Number of platform crossings. (H) Nesting scores from the nesting test. (I) Representative images from the nesting test. (J) Representative HPLC chromatograms of MT standard, mouse fecal samples from the MT intervention group, and brain tissue samples (axis transposed). (K) Representative heatmap of swimming trajectories in the water maze test. (L) Venn diagram of shared and unique OTUs (operational taxonomic units) across groups. (M) PCoA plot of fecal samples. (N) Firmicutes/Bacteroidota (F/B) ratio. (O) Phylum-level microbial composition and relative abundance. (P) ACE index, Chao-1 index, Shannon index, and Simpson index are used to assess gut microbial richness and evenness. (Q) Comparative analysis of relative abundances of differentially expressed gut microbiota across groups. Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, and the # group with the Model group, respectively).

Figure 8

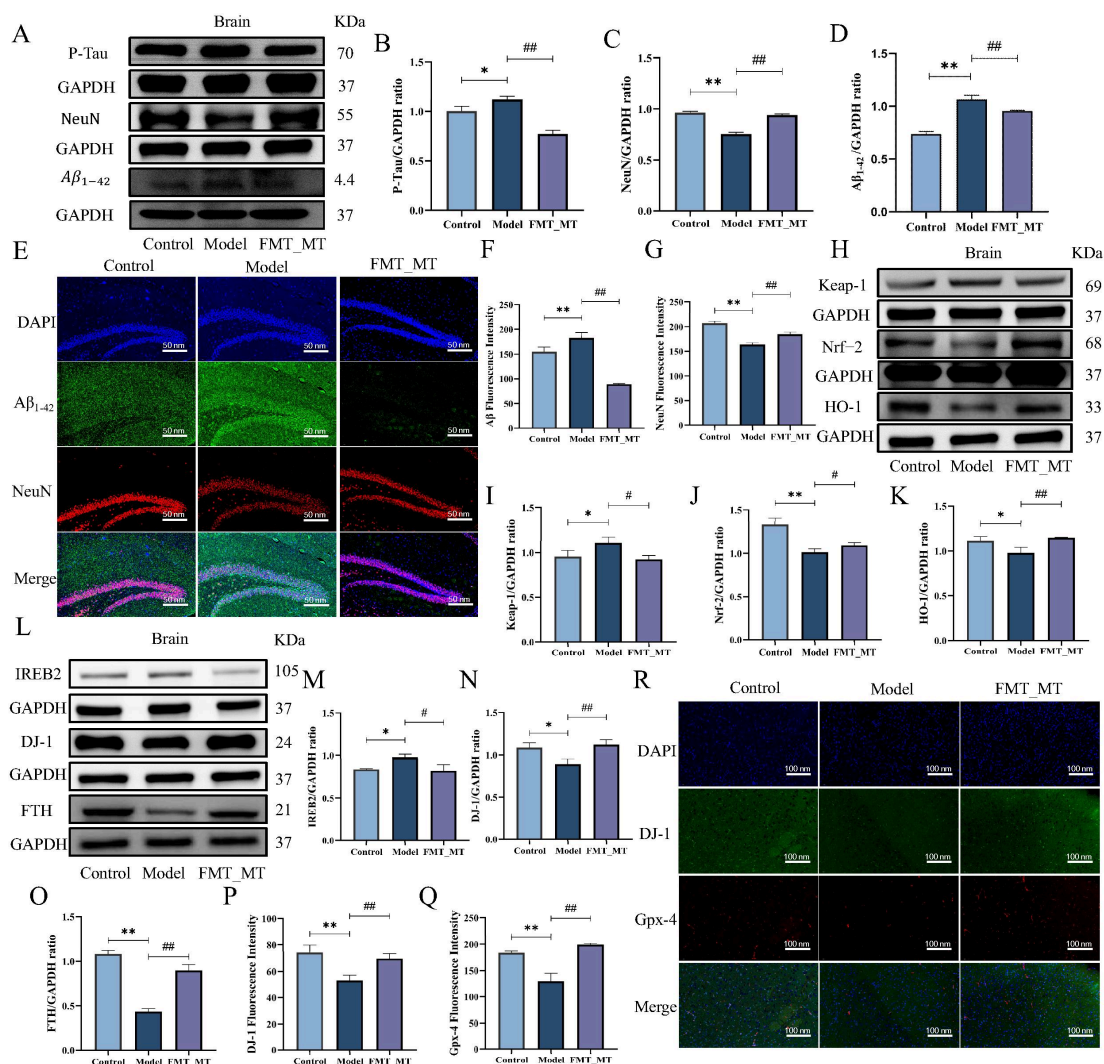


Figure 8

MT-remodeled gut microbiota mediate the regulatory role of MT in ferroptosis in AD. (A-D)

Representative Western blot images and grayscale analysis of P-Tau, NeuN, and Aβ₁₋₄₂ in brain tissue (with GAPDH as an internal control). (E) Representative immunofluorescence images of Aβ and NeuN in brain tissue (scale bar: 50 μm). (F-G) Quantitative analysis of Aβ and NeuN fluorescence intensity. (H-K) Representative Western blot images and grayscale analysis of Keap-1, Nrf-2, and HO-1 in brain tissue

(with GAPDH as an internal control). (L-O) Representative Western blot images and grayscale analysis of IREB2, DJ-1, and FTH in brain tissue (with GAPDH as an internal control) (P-Q) Quantitative analysis of the fluorescence intensity of DJ-1 and Gpx-4. (R) Representative immunofluorescence images of Gpx-4 and DJ-1 in brain tissue (scale bar: 100 μ m). Data are presented as mean SD (n = 6). *, # P < 0.05; **, ## P < 0.01 (comparing the * group with the Control group, and the # group with the Model group, respectively).

Figure 9

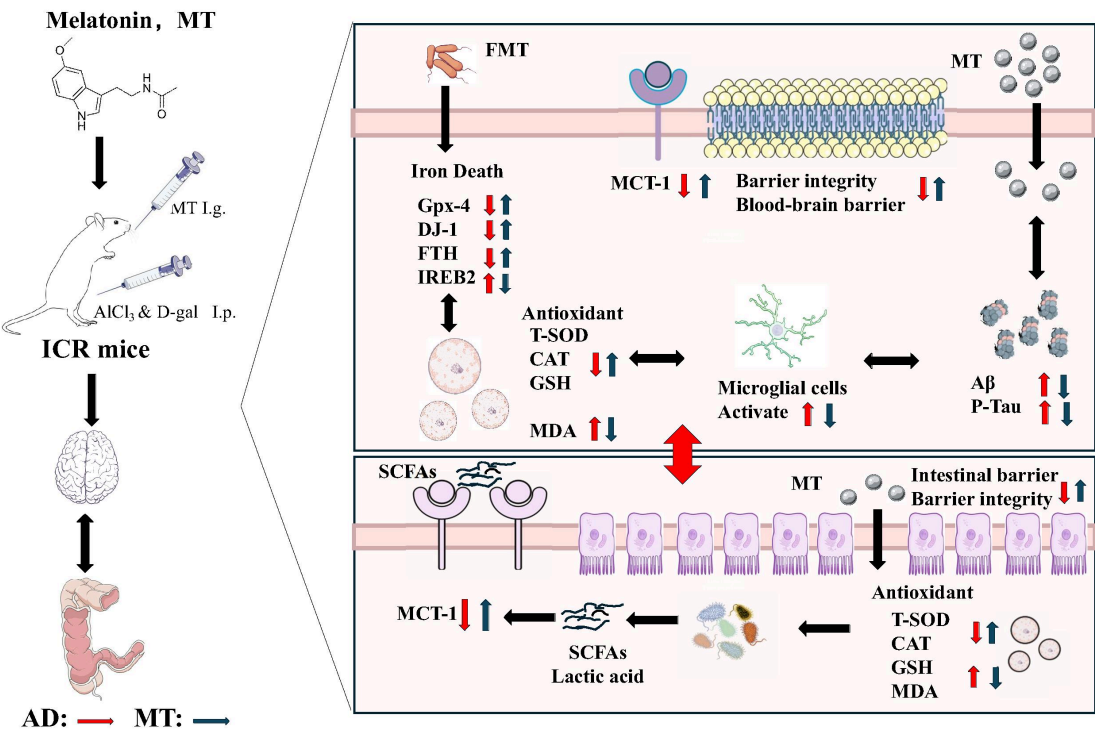


Figure 9

Schematic diagram illustrating the mechanism by which gut microbiota-mediated MT inhibition of ferroptosis improves AD pathology and AD colon comorbidity with iron overload.

Supplementary Files

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- [OriginalWesternBlots.pdf](#)
- [GraphicalAbstract.pdf](#)