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Lacticaseibacillus rhamnosus KF7 ameliorates cognition impairments via prolactin signaling modulation in senescence mice induced by D-galactose

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ABSTRACT: Aging-related cognitive decline poses a significant public health challenge with limited preventive options. Probiotics show promise in ameliorating aging-related diseases via the microbiota-gut-brain axis (MGBA). This study demonstrated that *L. rhamnosus* KF7 protected against D-galactose-induced aging in mice. *L. rhamnosus* supplementation significantly improved cognitive deficits in these mice. Crucially, *L. rhamnosus* reduced cerebral neuroinflammation markers (IL-1 β , IL-6) and oxidative stress (MDA), inhibited monoamine oxidase (MAO) activity, and restored neurotransmitter balance. Transcriptomics revealed *L. rhamnosus* KF7 modulated Prolactin signaling, and then enhanced NRF2 expression. Metabolomics and 16S rRNA sequencing showed *L. rhamnosus* KF7 restored microbial metabolic disturbances, increased the abundance of *Lactobacillus murinus*, and elevated levels of the Indole-3-lactic acid (ILA, a neuroprotective metabolite) in gut. These findings provided compelling evidence that *L. rhamnosus* KF7 conferred protection against cognitive impairment in this aging model, mediated via the MGBA. Mechanically, *L. rhamnosus* KF7 enhances the production of gut microbiota-derived ILA, which activates the prolactin signaling pathway in pituitary and then upregulates the expression of NRF2 in hippocampus, ultimately ameliorating multiple indicators associated with D-galactose-induced cognitive impairment. The study established a foundation for developing probiotics against aging-related cognitive decline.

Keywords: Aging; Cognitive impairment; microbiota-gut-brain axis; *Lacticaseibacillus rhamnosus* KF7; Prolactin; Indole-3-lactic acid.

1. Introduction

Aging is an irreversible biological process characterized by progressive physiological decline^[1]. From the year 2000 to 2050, the global population aged 60 years old and over will be tripled, increasing from 600 million to 2 billion^[2]. This demographic shift intensifies the burden of aging-related neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease, in the aging population worldwide and their quality of life^[3]. In recent years, a consensus has emerged that the structural imbalance of the gut

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microbiota is an important factor in inducing the onset of Alzheimer's disease [4, 5]. However, no drug so far can effectively stop the development of AD in patients [6]. It is crucial to find effective treatments to ameliorate aging-related neurodegenerative diseases.

The microbiota-gut-brain axis (MGBA) is a bidirectional communication network linking the gut microbiota and central nervous system (CNS), making it an actionable target to ameliorate the development and progression of neurodegenerative diseases [7]. A recent review has highlighted the contribution of the disturbances in the MGBA to the development of AD [8]. Evidence indicates that certain probiotics could enhance cognitive function in AD model or patients via MGBA, such as *Clostridium butyricum* [9], *Bifidobacterium longum* [10] and *B. breve* [11]. Qiao et al. [12] demonstrated that *Lactobacillus casei* improved cognitive decline in D-galactose/aluminum chloride-induced AD model mice by promoting the synthesis of bacterial short-chain fatty acids (SCFAs) in gut. *Lacticaseibacillus rhamnosus* is an important member of the former genus *Lactobacillus* [13]. It could improve the depressive and anxiety-like behaviors in animal models through MGBA [14, 15]. *L. rhamnosus*-derived exopolysaccharide can improve oxidative stress and regulate gut microbiota in mouse aging model. However, the mechanism by which *L. rhamnosus* protects aging mice from MGBA remains unclear.

Injections of D-galactose, usually employed to create animal models of aging, impair neurogenesis in the hippocampus in mice, which closely resembles the state of natural aging [16]. Initially, *L. rhamnosus* KF7 was screened for its strong anti-oxidant and anti-MAO activities *in vitro*, suggests that it may have potential neuroprotective effects [17, 18]. In this study, we assessed the functionality of *L. rhamnosus* KF7 in improving age-related cognition impairment in D-galactose induced aging mice. Behavioral tests to evaluate the mice's mental state, assays of brain inflammatory factors, oxidative stress markers, neurotransmitter levels, and transcriptional profiles were performed to reveal the neural mechanism by which *L. rhamnosus* KF7 functions. Gut microbiome and metabolome were also detected to investigate the MGBA-mediated protective effects of *L. rhamnosus* KF7 in the D-galactose-induced aging mouse model. This study found that *L. rhamnosus* KF7 can improve learning and memory impairments induced by D-galactose, and the mechanism by which it exerts effects involves MGBA. It provides a theoretical basis for the application of *L. rhamnosus* KF7 in improving cognitive impairments associated with aging.

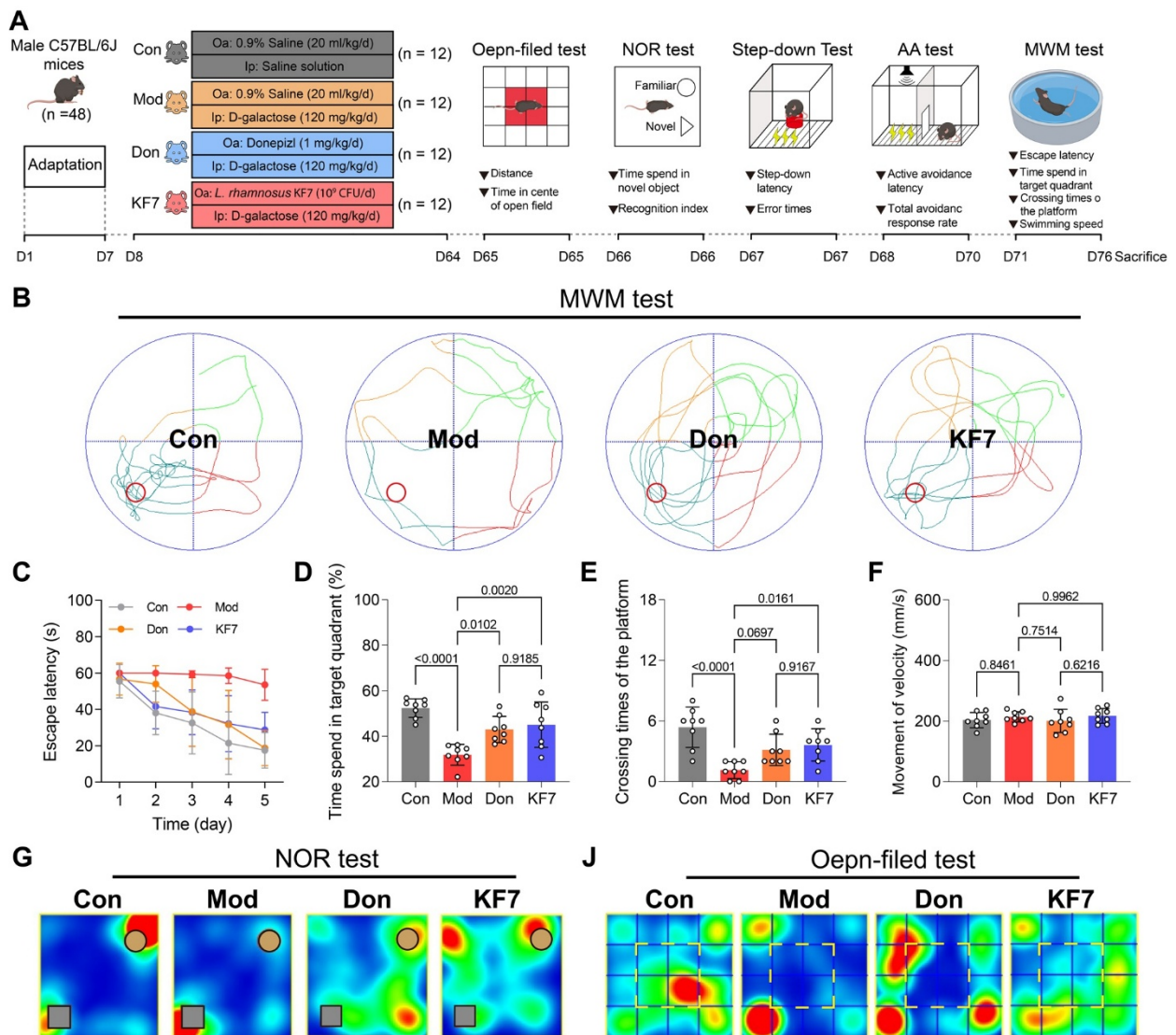
2. Materials and methods

2.1. Bacterial strains

L. rhamnosus KF7 (CGMCC No. 6430) was supplied by Bright Dairy & Food Co., Ltd. (Shanghai, China). The bacterial strain was activated on MRS (De Man, Rogosa, and Sharpe) agar at 36 °C in an anaerobic chamber (Bugbox, Ruskinn Com., England) with an atmosphere of N₂: H₂: CO₂=80:10:10 for 48 h and then a colony was transferred to MRS broth (Merck, German) at 37 °C for 16-18h. The cells were collected via centrifugation at 4 °C, 8000×g for 10 min and the cell pellets were adjusted to 5×10⁹ colony-forming unit (CFU)/mL with saline solution.

2.2. Animals and treatment

48 specific-pathogen-free (SPF) seven-week-old male C57BL/6J mice were purchased from Jiangsu Huachuang Sino Pharmaceutical Technology Co., Ltd (Jiangsu, China), and allowed a 1 week acclimatization. All experimental procedures received approval from the Ethics Committee of the Laboratory Animal Center of Tongji University School of Medicine (Ethics number: TJ-HB-LAC-2023-41). The mice were randomly assigned to four groups (n = 12 per group). The control (Con) group was administered 0.9% saline solution via intraperitoneal injection, while the model (Mod), *L. rhamnosus* KF7 (KF7), and donepezil (Don) groups received daily injections of 240 mg/kg of D-galactose (cat No. D810319, Macklin, Shanghai, China). Additionally, the KF7 group was administered 0.2 mL of a 5×10^9 CFU/mL *L. rhamnosus* KF7 solution daily via oral gavage, while the Don groups was administered 1.0 mg/kg donepezil (cat no. HY-14566, MedChemExpress, NJ, USA) solution daily via oral gavage. The Con and Mod groups continued to receive equal volume of 0.9% NaCl. The weights of the mice were recorded every 7 days to correct for the dose. The experimental procedure is shown in **Figure 1A**. The behavioral tests lasted 13 days, and drug administration was performed synchronously during the tests.



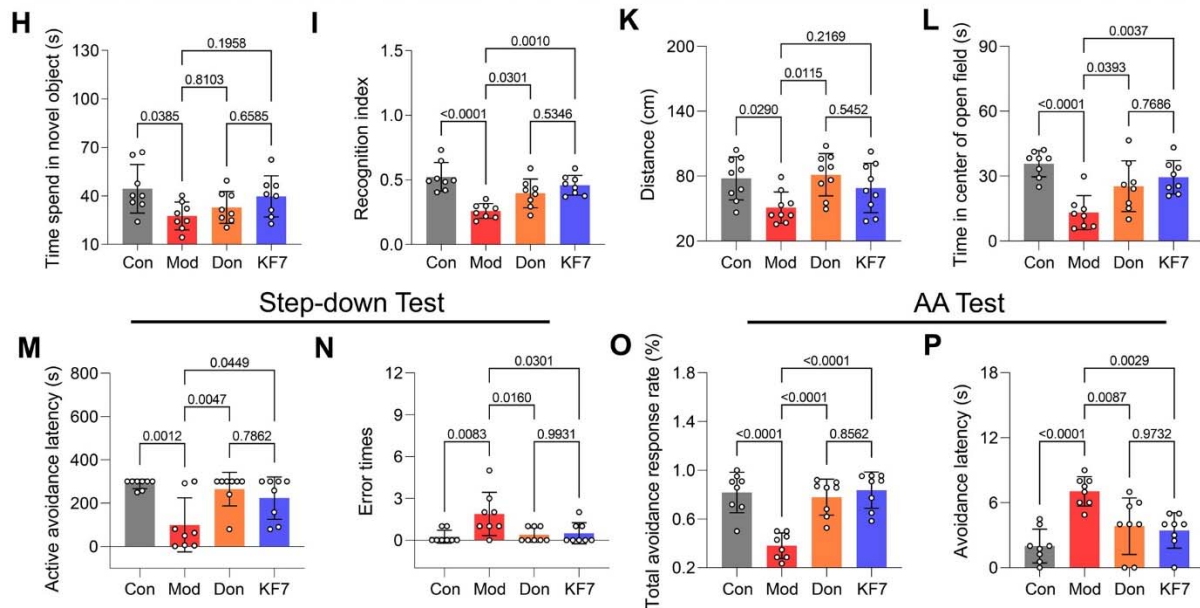


Figure 1. Effects of *L. rhamnosus* KF7 supplementation on D-galactose-induced cognitive impairment in mice. (A) Experimental timeline of this study. Oa: orally administered; Ip: intraperitoneal injection; NOR: novel object recognition; AA: Step-through active avoidance; MWM: Morris water maze. (B) Representative swimming paths of mice on the test day in MWM test. (C) Escape latency during training days in MWM test. (D) Time spend in target quadrant, (E) Crossing times of the platform, and (F) Movement of velocity on the test day in MWM test. (G) Representative trajectory heatmaps of mice in NOR test. (H) Time spent in novel object and (I) Recognition index of mice in NOR test. (J) Representative trajectory heatmaps of mice in open-field test. (K) Distance and (L) Time in center of open field in open-field test. (M) Active avoidance latency and (N) Error times in step-down test. (O) Total avoidance response rate and (P) Avoidance latency in AA test. The *p*-value is directly indicated on the bar chart, The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with *n* = 8 per group.

2.3. Behavioral Testing

2.3.1. Open field test

On the 65th day, the mice were placed in an open space to acclimatize for 10 min and then placed in a box (length 50 cm × width 50 cm × height 40 cm) for the experiment. Each mouse was placed in the center of the open field apparatus and allowed to explore freely for 5 min. Parameters were recorded by a computer connected camera, including the distance traveled by the mouse, average speed, rest time, and distance traveled in the central region.

2.3.2. Novel object recognition (NOR) test

On the 66th day, the mice with different treatment were assessed for their behavior in **NOR**. The apparatus utilized in NOR test was consisted of a 50 × 50 × 20 cm opaque, rectangular box with a camera hang on the ceiling (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China), Cylinders and cubes made of the same material (wood) were used as the objects. The NOR test was performed in three stages: the adaptation stage, the training stage for familiar object memory, and the recognition stage for novel objects. On the first stage, mouse was individually placed in the box without any object and allowed to explore freely for 3 min. At the end of stage, after the mouse was removed, the box was wiped with 75% ethanol to remove the residual odors of mice. After an interval of 2 hours, the training of mice proceeded to the next stage. On second stage, two identical objects were placed and fixed with double-sided tape in the

box, and the mouse was placed in the device to freely explore for 5 min, after which the mouse was removed and waiting for 1 hour. On the final stage, in the same box, one of the previous objects was replaced with a novel object, and the mouse was allowed to explore the device for 5 min. The time spent by individual mouse exploring each object was recorded.

2.3.3. Step-down type passive avoidance test

On the 67th day, the step-down avoidance test was conducted. The apparatus includes a plastic box with a stainless steel grid floor and a foam platform. At the beginning of the test, mice were placed in the apparatus to adapt for 5 min. Then electric current was delivered and maintained for 5 min. During the process, mice will jump onto the platform to avoid electric shocks. The parameters, including the latency to step down on the grid for the first time, the number of errors within 5 min and the number of electrical shocks within 5 min, were recorded for each mouse.

2.3.4. Active avoidance (AA) test

The step-through active avoidance test was performed by using a two-compartment shuttle box (each $136 \times 153 \times 250$ mm) adjacent to each other through an arch door (3 cm in diameter). The floor of the compartments was composed of 2 mm stainless-steel rods spaced 1 cm apart. In an acquisition trial, mice was initially placed in one compartment facing away from the door and allowed to adapt for 5 min. After acclimatization, a buzzer and a light were delivered in the compartments for 5 s. If the mouse stopped still in this compartment, then it was shocked by an electric foot current (10–20 mA, 10–15 Hz) through the grid floor in this compartment until it escaped into the other compartment. The average inter-trial interval was 60 s. Each test contained 20 combines of buzzer and light and foot electric shocks. Each mouse was trained for 20 trials per day for 2 consecutive days. At the last day, the final test results were recorded: (i) active avoidance response latency; (ii) escape response latency.

2.3.5. Morris water maze (MWM) test

The MWM apparatus consisted of a circular tank (diameter, 120 cm; depth, 50 cm) filled with water and painted black to hide the platform (diameter, 10 cm). Different visual cues were placed at four locations: north, south, east, and west. The hidden platform was placed in the southeast center. In the training section (days 1–5), mice were placed in the MWM apparatus and allowed to swim freely for 60 s until they found the hidden platform. If a mouse did not find the platform within 60 s, it was gently placed on it. Thirty seconds after a mouse found the platform, it was returned to the home cage. Training was conducted three times a day for consecutive 5 days. In the probe section, mice were allowed to swim freely for 60 s without using a hidden platform. The time and distance that the mice stayed in the target quadrant, the number of target crosses, and the velocity were analyzed using the VsiuTrack animal behavior analysis software (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China).

2.4. Biochemical analysis and enzyme-linked immunosorbent assay (ELISA)

The levels of Malondialdehyde (MDA, cat no. A003-1, Nanjing Jiancheng Institute of Biotechnology, Nanjing, China), Monoamine oxidase (MAO, cat no. A034-2-1, Nanjing Jiancheng Institute of Biotechnology, Nanjing, China), Indole-3-lactic Acid (ILA, cat no. MM-46497M2, MEIMIAN, Jiangsu, China), Interleukin 1 Beta (IL-1 β , cat no. E-EL-H0149, Elabscience, Wuhan, China), Interleukin 6 (IL-6, cat no. E-EL-M0044, Elabscience, Wuhan, China), Tumor Necrosis Factor Alpha (TNF- α , cat no. E-EL-M3063, Elabscience, Wuhan, China), and Interleukin 10 (IL-10, cat no. E-EL-M0046, Elabscience, Wuhan, China) were detected by corresponding assay kits. The mice brains of each group were pretreated with 1 $\times$ phosphate buffer saline (PBS, cat no. G4202, Servicebio, Wuhan, China) and homogenized. The homogenate was centrifuged at 5000 $\times$ g at 4 $^{\circ}$ C for 10 min. The supernatant was stored at -80 $^{\circ}$ C until the assay. All the assays were operated according to individual kit manufacturer's protocols and detected with a Universal Microplate Spectrophotometer (Bio-Rad, Hercules, USA).

2.5. Detection of neurotransmitters

LC-MS/MS was employed to analyze 46 neurochemicals, including GABAergic system (glutamate, glutamine and γ -amino-butyric acid (GABA)), the dopaminergic system (DOPA, dopamine, norepinephrine, 3-methoxytyramine, tyramine and normetanephrine), serotonergic and kynurenergic system (5-hydroxyindoleacetic acid, serotonin and 5-hydroxy-L-tryptophan) and the cholinergic system (acetylcholine), etc (**Supplemental Table 1**). The neurotransmitters were determined according to previous study ^[19]. Briefly, mouse brain (n = 6 per group) were weighed and then homogenized in 100 μ L of 1% formic acid. Then 400 μ L acetonitrile solution containing 1% formic acid was added, and vortexed for 60 s. Low temperature ultrasound was then conducted for 30 min twice. Treated samples were then centrifuged for 20 min at 14,000 $\times$ g and the supernatant was freeze-dried and stored at -80 $^{\circ}$ C until further analysis.

The neurotransmitters were quantitatively determined using LC-MS/MS. Mobile phase A was water (25 mM ammonium acetate) containing 0.1% Formic acid (FA), and mobile phase B was acetonitrile containing 0.1% FA. A gradient 0–18 min, 90%–40% B, 18–18.1 min 40%–90% B, and re-equilibration at 90% from 18.1–23 min. The flow rate was set at 300 μ L /min, and the injection volume was 2 μ L. A 5500 QTRAP mass spectrometer (AB SCIEX) was used for mass spectrometry operated in the positive MRM mode. Operating conditions were as follows: IonSapary voltage floating (ISVF), 5000 V; source temperature, 300 $^{\circ}$ C; ion source gas1 (Gas1), 60psi; ion source gas2 (gas2), 60 psi; curtain gas, 30 psi. Quantification was made by reference to calibration curves obtained from individual neurotransmitter standards, and concentrations were expressed as ng/mg wet weight. Heatmaps were made using TBtools-II (version 2.310) ^[20].

2.6. RNA sequencing

The RNA-seq was performed according to previous study ^[21]. Briefly, total RNA from mouse brain (n = 3 per group) was purified with an RNApure Fast Tissue and Cell Kit (cat no. CW0599S, CWBIO, Beijing, China), and the quality of the RNA was checked with an ND-1000 (NanoDrop), a Bioanalyzer 2100 (Agilent), and agarose gel electrophoresis. The mRNA with polyadenylation was trapped by magnetic beads

(cat no. 88802, Thermo Fisher, Waltham, USA) and fragmented by a Magnesium RNA Fragmentation Module. The sequencing library with a fragment size of 300 ± 50 bp was constructed and sequenced using NovaSeq X Plus in PE150 sequencing mode.

The raw data were processed using Cutadapt-1.9 software to remove low-quality splice sequences and duplicate sequences. The clean data were compared to the genome using HISAT2-2.0.4 (<https://daehwankimLab.github.io/hisat2/>). FPKM (Fragments per kilobase of transcript per million fragments mapped) was calculated as $\text{FPKM} = \frac{\text{total_exon_fragments}}{\text{mapped_reads (millions)} \times \text{exon_length (kB)}}$ using the ballgown package. Differential expression analyses of the four groups were performed using the DESeq2 R package (<http://www.bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html>) based on the negative binomial distribution (1.16.1 version), and genes with $\log_2|\text{fold change}| \geq 1$ and $P < 0.05$ were defined as differentially expressed genes (DEGs) and subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using KOBAS (<http://bioinfo.org/kobas>) based on ORA (Over-Representation Analysis) and GSEA (Gene Set Enrichment Analysis).

2.7. Metabolomics

The fecal metabolome of mouse ($n = 4$ per group) were performed according to previous study. Non-targeted metabolomics experiments were conducted according to previous studies [22]. The extraction simplification step and conditions were as follows: 1 mL 70% (v/v) precooled (4°C) methanol was added to a 2.0 mL microcentrifuge tube with a 100 mg sample of mouse feces and stored at 4°C overnight for extraction, centrifuged at $10,000 \times g$ for 10 min and then the supernatants were collected. The supernatant was subsequently absorbed by CNWBOND Carbon-GCB SPE Cartridge (250 mg, 3 mL, ANPEL, Shanghai, China). The adsorbed compounds were eluted using toluene-acetone mixture (3:7, v/v), and dried under vacuum. Sample extracts were analyzed using an LC-ESI-MS/MS system. The identified metabolites were annotated using HMDB (<https://hmdb.ca/>) and the KEGG compound database (<http://www.kegg.jp/kegg/compound/>). The annotated metabolites were subsequently mapped to the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>). Significantly differentially expressed metabolites between groups were determined by a VIP (variable importance in projection) ≥ 1 and a $p\text{-value} \leq 0.05$. VIP values were extracted from the orthogonal partial least squares discriminant analysis (OPLS-DA) results, which also contained score plots and permutation plots and were generated using the R package MetaboAnalystR (version 1.0.1). Significantly enriched pathways were identified with a hypergeometric test's P value (< 0.05) for a given list of metabolites.

2.8. 16S rRNA gene sequencing

16S rRNA gene sequencing was conducted as described in previous study [23]. Briefly, genomic DNA was extracted from the fecal samples from mice using a Bacteria Genomic DNA Kit (cat no. CW0552, CWBIO, Beijing, China). 16S rRNA sequencing was performed using an Illumina system (Illumina Corp., CA, USA). Sequencing and bioinformatics analysis were performed by Majorbio Bio-Pharm Technology

Co., Ltd (Shanghai, China). USEARCH software (<https://www.drive5.com/usearch>) was used to identified operational taxonomic units (OTUs). The microbial diversity was assessed by PCA and PCoA and microbial dysbiosis Index (MDI) analysis. Linear discriminant analysis effect size (LEfSe, <http://galaxy.biobakery.org/>) was conducted to evaluate the differences in species abundance across the groups. PICRUST2 (<http://huttenhower.sph.harvard.edu/galaxy>) was conducted to predict the functional differences among the various bacterial communities.

2.9. Reverse transcription-polymerase chain reaction (RT-qPCR)

RNA extraction and cDNA retro-transcription were performed according to the above methods. All primers for qRT-PCR were designed with Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) according to the corresponding sequences and are listed in **Supplemental Table 2**. All primers used spanned an exon–exon junction. The 20 µL reaction volume contained the following components: 10 µL of Universal Blue SYBR Green qPCR Master Mix (cat no. G3328, Servicebio, Wuhan, China), 0.4 µL each of forward and reverse primers, 2 µL of cDNA, and 7.2 µL of nuclease-free water. The qRT-PCR analyses were carried out using a two-step method, and the cycling parameters were as follows: 94 °C for 30 s, 40 cycles of denaturation (94 °C, 5 s), and annealing (60 °C, 30 s). All reactions were performed in duplicate or in triplicate with β-actin or GAPDH as the internal reference, and the relative mRNA expression data were calculated using the $2^{-\Delta\Delta Ct}$ method and were presented as the mean ± standard deviation (SD).

2.10. Data analysis

The statistics were presented as mean ± standard error of the mean (SEM) and each hollow dot on the bar represents the measurement of a sample. The correlation coefficient analysis was performed using the R package with the Spearman correlation method, and the parameters for correlation were established as follows: a coefficient greater than 0.35 or less than -0.35, along with a *p-value* of less than 0.05. The statistical analysis involved used one or two-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni multiple comparison tests as necessary. Each experiment was conducted at least three times. Significance was determined as $P < 0.05$. The *p-value* value was directly marked on the graph.

3. Results and Discussion

3.1. Administration of *L. rhamnosus* KF7 alleviated D-galactose-induced cognitive impairment in mice

Cognitive function plays a critical role in the daily lives of older adults, as powers related to learning and memory tend to decline with advancing age ^[24]. Research suggests that D-galactose can induce cognitive decline and memory deficits in mouse models, primarily through mechanisms involving oxidative stress and neuroinflammation ^[25]. To evaluate the efficacy of *L. rhamnosus* KF7 on cognitive dysfunction *in vivo*, we administered *L. rhamnosus* KF7 or Donepezil to D-galactose-induced aging mice for eight weeks (**Figure 1A**). The MWM revealed that male mice in the Mod group exhibited a disordered trajectory and spent more time finding the platform, both of which improved after the administration of *L. rhamnosus* KF7

or Donepezil during training days (**Figure 1B**). Statistically, the mean escape latency was significantly longer in the Mod group than in the Con group ($P < 0.05$, **Figure 1C**). Conversely, Don and KF7 groups presented significantly shorter mean escape latencies than did the Mod group ($P < 0.05$). Compared with those in the Mod group, the Don or KF7 group spent significant more time in the target quadrant and crossed the platform quadrant more times at a same swimming speed ($P < 0.05$), whereas the *L. rhamnosus* KF7 was equivalent to Donepezil ($P > 0.05$, **Figure 1D-F**). In the NOR test, mice in the Mod group tended to explore familiar object compared with novel object, which was reversed in the Don and KF7 groups (**Figure 1G**). The decreases in the time spent on the novel object and recognition index induced by D-galactose was reversed by the administration of *L. rhamnosus* KF7 or Donepezil ($P < 0.05$, **Figure 1H-I**). In the open field trajectory test, it was observed that Donepezil significantly reversed the reduction in the moving distance and the time spent in the center area in D-galactose induced aging mice, while *L. rhamnosus* KF7 only reversed the latter ($P < 0.05$, **Figure 1J-L**). In the step-down test, there was no significant difference in the avoidance latency and the error times among the groups of mice on the first day (**Supplemental Figure 1**). After 48 hours, the Mod group mice showed a shorter avoidance latency and more error times compared to Con group mice. In contrast, in the Don and KF7 groups, this trend was significantly reversed ($P < 0.05$, **Figure 1M-N**). The Mod group mice showed a reduced avoidance response rate and higher avoidance latency during the AA test as compared to the Con group mice ($P < 0.05$, **Figure 1O-P**). However, the Don and KF7 groups showed significantly higher response rate and lower avoidance latency error times ($P < 0.05$). Similarly, *L. rhamnosus* GG supplementation can enhance the performance of mice in cognitive-related behavioral experiments, such as the open field and NOR tests [26]. The mixture of *L. rhamnosus* and *B. lactis* also demonstrated improved performance in NOR and MWM test in the senescence-accelerated-mouse-prone 8 (SAMP8) mice [27].

3.2. *L. rhamnosus* KF7 reduced neuroinflammation and oxidative stress induced by D-galactose in mice.

D-galactose can induce neuroinflammation and oxidative stress in the brains of mice [28]. High concentration of D-galactose causes oxidative stress through promoting generation of advanced glycation end product (AGEs) and MDA, while activates the nuclear translocation of NF- κ B and transcription of inflammatory-encoding genes and then induces neuroinflammation [29]. Therefore, we first assessed the mRNA and protein levels of inflammatory factors in the brain tissues of mice. The mRNA of the inflammatory cytokine-encoding genes *Il1beta*, *Il6*, and *Tnfa* were significantly higher in Mod group in comparison to the Con group ($P < 0.05$, **Figure 2A-C**). The administration of Donepezil or *L. rhamnosus* KF7 led to a significant decrease in the mRNA level of the above genes ($P < 0.05$). The transcriptional levels of the anti-inflammatory cytokine-encoding gene *Il10* in the brain tissues of the Don and KF7 groups were significantly higher than those in the Mod group ($P < 0.05$, **Figure 2D**). The protein levels of inflammatory factors further supported the results obtained in mRNA level ($P < 0.05$, **Figure 2E-H**). Aging elevates pro-inflammatory factors like IL-1 β , TNF- α , etc, consistent with our findings [30]. The hippocampus is particularly susceptible to neuroinflammatory responses. Pro-inflammatory cytokines within the

hippocampus have been shown to impair memory consolidation and disrupt cognitive function by interfering with key mediators involved in hippocampal memory formation [31]. Pang et al. [32] demonstrated that *L. rhamnosus* GG could also regulate the transcription and translation of these inflammatory factors in the cognitive impairment model induced by sleep deprivation.

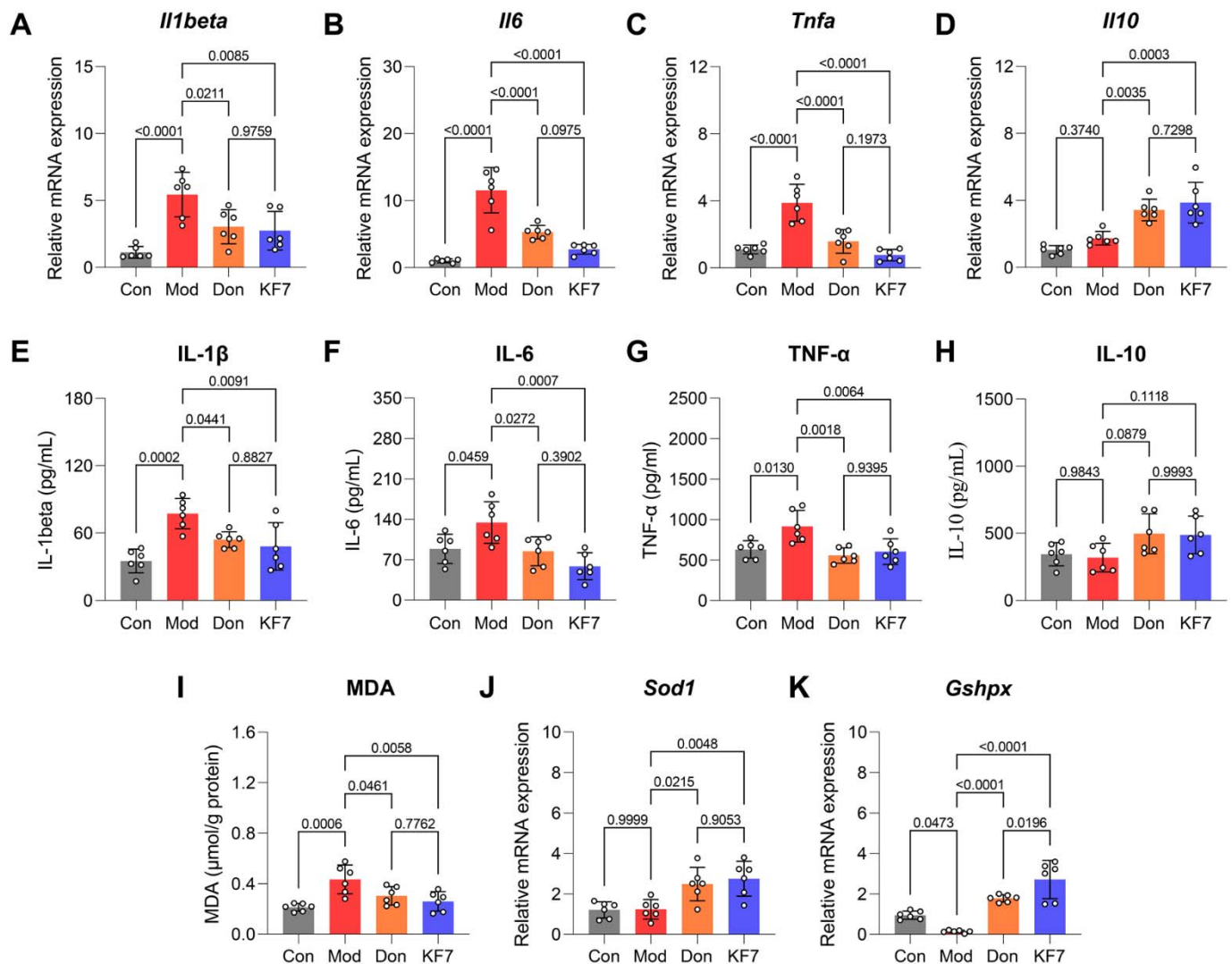


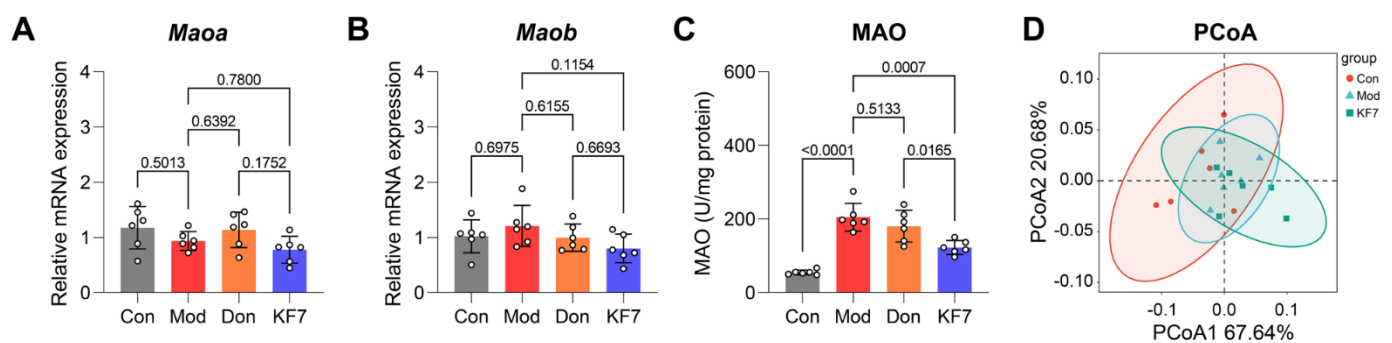
Figure 2. Effects of *L. rhamnosus* KF7 supplementation on D-galactose-induced neuroinflammation and oxidative stress in mice. The mRNA levels of (A) *Il1beta*, (B) *Il6*, (C) *Tnfa*, and (D) *Il10* in brains of mice from Con, Mod, Don, and KF7 groups detected by RT-qPCR. The protein levels of (E) IL-1 β , (F) IL-6, (G) TNF- α , and (H) IL-10 in brains of mice from four groups detected by ELISA. (I) The level of MDA in brains of mice from four groups. The mRNA levels of (J) *Sod1* and (K) *Gshpx* in brains of mice from four groups detected by RT-qPCR. The *p*-value is labeled on the bar chart. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with $n = 6$ per group.

The overall oxidative stress of the brain is an important symptom in patients with AD [33]. MDA is the final product of lipid peroxidation reaction. Increase in MDA levels typically indicated the enhancement of oxidative stress and associated with various neurological disorders [34]. Here, we found that the accumulation of MDA in the mouse brain induced by D-galactose was significantly reversed after the administration of Donepezil or *L. rhamnosus* KF7 ($P < 0.05$, Figure 2F). The level of SOD1 in the brains of AD patients decreased significantly, while the levels of other SOD isozymes did not show any obvious changes [35]. Autopsy and *in vivo* studies consistently confirmed that the activity of GSHpx in the brains of AD patients

decreased and correlated with the severity of the AD ($R = -0.68$, $P < 0.01$) [36]. The supplement of *L. rhamnosus* KF7 could also significantly increase the mRNA level of antioxidant related genes *Sod1* and *Gshpx* (**Figure 2G-H**). It exhibited significantly superior efficacy compared to Donepezil in terms of *Gshpx* transcription ($P < 0.05$).

3.3. *L. rhamnosus* KF7 changed the expression of neurotransmitters especially indole-3-lactic acid (ILA) and Kynurenine in the brains of D-galactose-treated mice

Monoamine oxidase-B (MAO-B) has recently emerged as a potential therapeutic target for AD [37]. In the astrocytes surrounding the amyloid plaques in the brains of AD patients, the activity of MAO-B increased by 2 to 3 times [38]. Our previous work reported that the fermentation supernatant of *L. rhamnosus* KF7 possessed strong inhibitory activity to MAO *in vitro* [17]. In present study, we assayed the effects of *L. rhamnosus* KF7 on the transcription and translation of MAO in the brains of D-galactose-treated mice. The RT-qPCR analysis revealed comparable transcriptional levels of *Maoa* and *Maob* across all mouse brain samples ($P > 0.05$, **Figure 3A-B**). The Mod group exhibited significantly elevated MAO activity in brain tissue compared to those in Con group ($P < 0.05$, **Figure 3C**). *L. rhamnosus* KF7 treatment significantly reduced cerebral MAO levels ($P < 0.05$), whereas donepezil showed no comparable effect ($P > 0.05$). These results suggest that the increased MAO activity in the Mod group mice may not be caused by MAO gene transcription alone, but may be caused by post-translational modifications or differences in enzyme activators. MAO inhibitors were reported to provide beneficial neuroprotective effects for AD therapy [39]. Although identified several decades ago, this target continues to attract significant attention for inhibitor development [40]. However, chemical MAO inhibitors frequently exhibit safety concerns. Developing safer strategies to cope with the age related cognition dysfunction remain a top challenge. Our team has continuously tried to isolate Lactobacilli exhibiting MAO inhibitory activity such as *Lacticaseibacillus casei* JH-23 from human microbiota, a pioneer strain documented with this property [41]. Later, we discovered *L. rhamnosus* KF7, which has a stronger inhibitory activity on MAO. In this study, we verified the MAO inhibitory ability of *L. rhamnosus* KF7 *in vivo* through a D-galactose-induced aging mouse model.



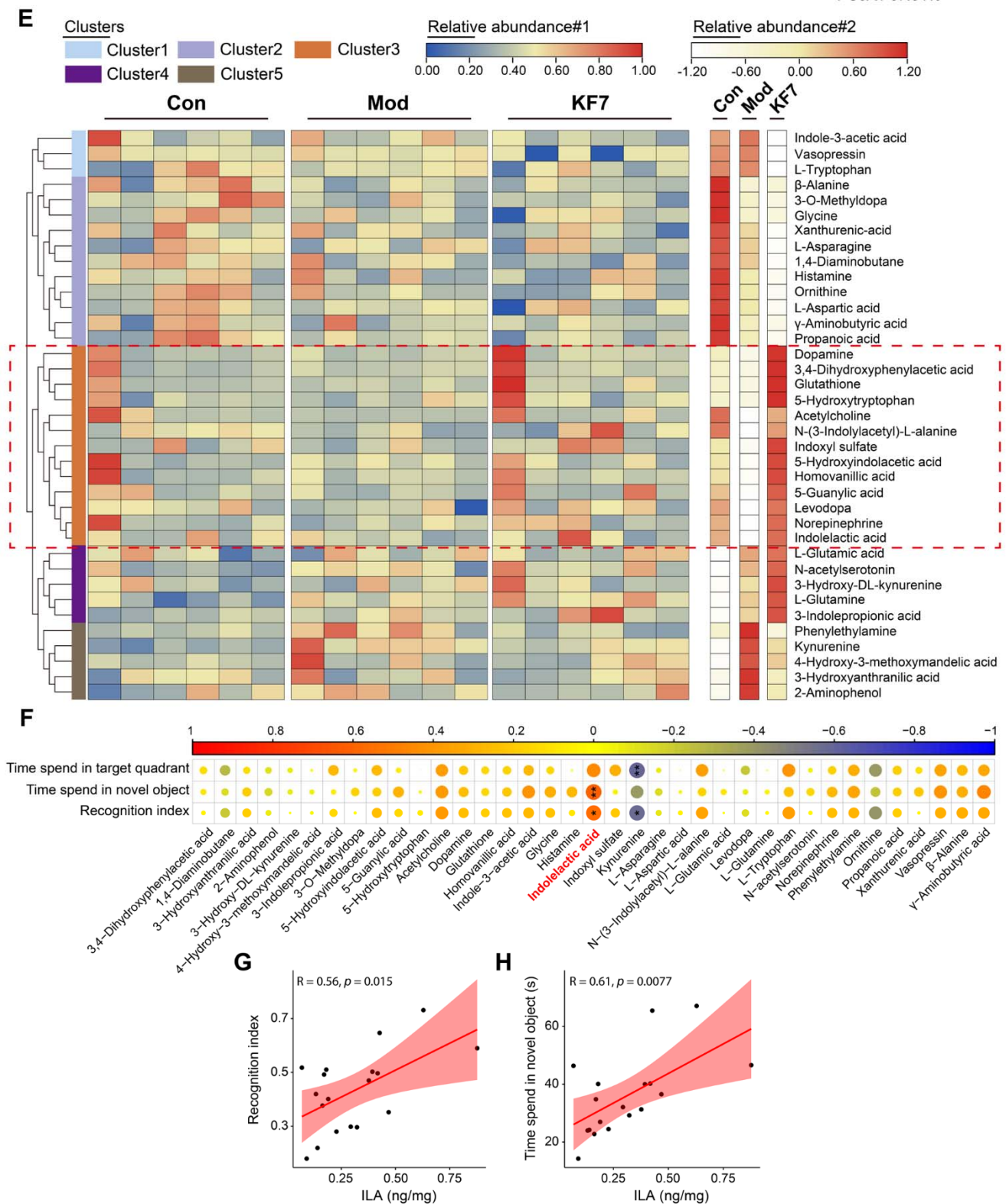


Figure 3. Effects of *L. rhamnosus* KF7 supplementation on MAO and neurotransmitter profile in D-galactose-induced aging mice. The mRNA levels of (A) *Maoa* and (B) *Maob* in brains of mice from four groups detected by RT-qPCR. (C) The activity of MAO in brains of mice from four groups. (D) PCoA of neurotransmitter profiles among brains of mice from Con, Mod, and KF7 groups. (E) Hierarchical clustering heatmap of 37 detectable neurotransmitters among brains of mice from three groups. The red dashed box refers to a group of neurotransmitters that decreased in the Mod group but showed a recovery trend in the KF7 group. (F) Correlation analysis of three behavioral indicators (Time spend in target quadrant (MWM), Time spend in novel object (NOR), and Recognition index (NOR)) and 37 detectable neurotransmitters ($n = 6$ per group). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Correlation scatter plots of (G) Recognition index and ILA level as well as (H) Time spend in novel object and ILA level. The p -value is labeled on the bar charts (A-C). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with $n = 6$ per group.

MAO is involved in the metabolism of various neurotransmitters, thereby influences key processes such as neural development in the brain [42]. To elucidate the changes in neurotransmitters after treatment with *L. rhamnosus* KF7 in D-galactose-treated mice, the LC-MS/MS assay was performed to analyze 46 neurochemicals (**Supplemental Table 1**). PCoA of 37 detectable neurotransmitters revealed similar profiles across Con, Mod, and KF7 groups (**Figure 3D**). However, cluster analysis showed five distinct neurotransmitter expression patterns across the three groups (**Figure 3E**). The heatmap analysis further revealed distinct variations in these neurotransmitter profiles. Among these metabolites, one subset exhibited lower levels in the Mod group but showed elevated concentrations in KF7 group relative to controls. The metabolites in this subset exhibited strong intercorrelation (**Supplemental Figure 2**). Correlation analysis showed that only two neurotransmitters indole-3-lactic acid (ILA) and Kynurenine—exhibited significant correlations with both the recognition index and the time spend in novel object or time spend in target quadrant in corresponding mice from three groups (**Figure 3F**). The correlation coefficients for ILA with the corresponding recognition index and time spent in novel object were 0.56 and 0.61, respectively (**Figure 3G-H**). ILA is important in regulating neurogenesis. Previous studies have shown that ILA levels decreased in patients with AD, and restoring ILA levels could alleviate the cognitive impairments AD patients [43]. Here, we found that the level of cerebral ILA was positively correlated with the cognitive level of the aging mice.

3.4. *L. rhamnosus* KF7 reversed the up-expression of genes encoding proinflammation cytokines in the brains of ageing mice induced by D-galactose

Transcriptomics enables comprehensive mapping of gene expression patterns, serving as a fundamental approach for elucidating drug mechanisms [44]. The transcriptional landscapes of the brain in Mod and KF7 mice were compared. PCA showed significantly different clustering of genes in Mod mice compared to that in KF7 mice (**Figure 4A**). A total of 73 up-regulated DEGs and 125 down-regulated DEGs were identified between Mod and KF7 group (**Supplemental Figure 3**). The GO enrichment analysis of DEGs indicates that a total of 190 BP terms, 38 MF terms, and 29 CC terms were significantly enriched (**Figure 4B**). In the clustering of significantly enriched BP terms, the largest module was associated with “response”, whereas the second largest module mainly corresponded to “regulation” (**Figure 4C**). ORA on KEGG pathways showed that the up-regulated DEGs were related to multiple signaling pathways, including the Prolactin (PRL) signaling and MAPK signaling pathway (**Figure 4D**). Down-regulated DEGs exhibited a stronger correlation with inflammatory signaling pathways, such as “Toll-like receptor signaling pathway”, “Cytokine-cytokine receptor interaction”, and “Staphylococcus aureus infection” (**Figure 4E**).

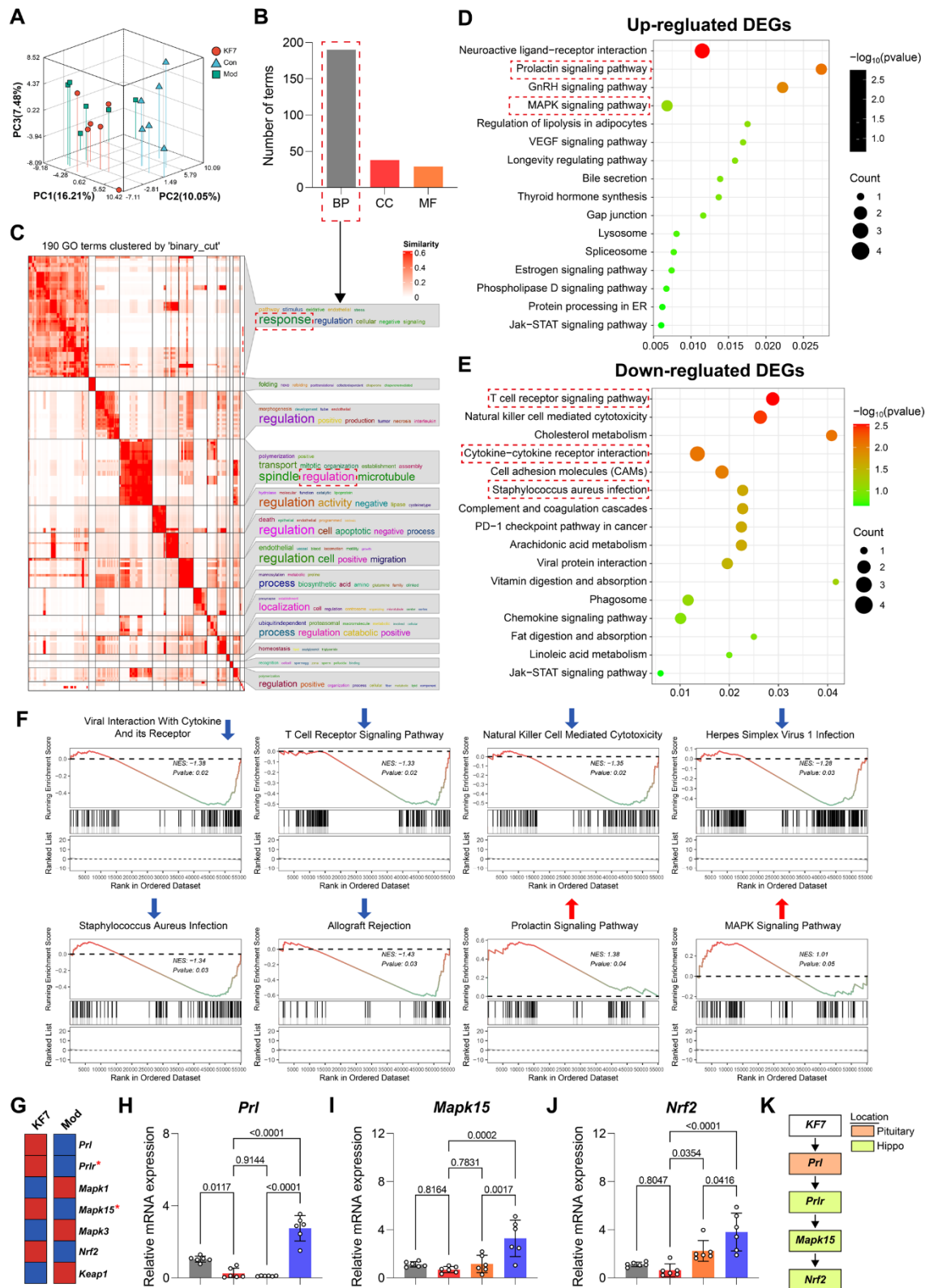


Figure 4. The effect of *L. rhamnosus* KF7 supplementation on the transcriptional profile of D-galactose-induced aging mice. (A) PCA of cereal transcriptomes of mice from Mod and KF7 groups. (B) Histogram of the BP, CC, and MF GO terms significantly enriched by the DEGs. (C) The overall visualization of the significantly enriched BP class GO entries. ORA of (D) up-regulated and (E) down-regulated DEGs based on the KEGG database. (F) GSEA of transcription profile based on the KEGG database. The blue arrow indicates that NES is less than 1, while the red arrow indicates that NES is greater than 1. (G) Heatmap of the average mRNA expression (based on counts) levels of key genes in the 'Prl-MAPK-NRF2' axis. The mRNA levels of (H) *Prl*, (I) *Mapk15* and (J) *Nrf2* in brains of mice from four groups detected by RT-qPCR. (K) The preliminary molecular mechanism underlying *L. rhamnosus* KF7-mediated attenuation of cognitive impairment. The *p*-value is labeled on the bar charts (I-K). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with *n* = 3 per group.

Further GSEA (gene set enrichment analysis) on KEGG pathways revealed that *L. rhamnosus* KF7 inhibited the inflammatory response induced by D-galactose (**Figure 4F**). The PRL and MAPK signaling pathways, identified through ORA as enriched by up-regulated DEGs, both showed significant up-regulation in the KF7 group in GSEA. The transcriptional levels of key genes (*Prl*, *Prlr*, *Mapk15*, and *Nrf2*) in the PRL, MAPK, and NRF2 pathways demonstrated increased expression in brain tissue following *L. rhamnosus* KF7 treatment (**Figure 4G**). The RT-qPCR results corroborated the differential expression patterns observed for these genes ($P < 0.05$, **Figure 4I-K**). Based on above results, the preliminary molecular mechanism underlying *L. rhamnosus* KF7-mediated attenuation of cognitive impairment was constructed (**Figure 4L**). PRL is a peptide hormone mainly secreted from the anterior pituitary gland [45]. In canonical signaling, PRL binding to prolactin receptor (PRLR) induces downstream signaling including JAK-STAT, AKT and MAPK pathways [46]. Activation of the MAPK pathway has been found to improve neuroinflammation [47], oxidative stress [48], and neurotransmitter disorders [49]. Mitogen activated protein kinase 15 (MAPK15), also known as Extra-cellular signal regulated kinase 7/8 (ERK7/8), is an atypical member of the MAP kinase family whose activation has been found to regulate nuclear factor erythroid 2-related factor 2 (NRF2) activity [50]. The activation of NRF2 can inhibit neuroinflammation and oxidative stress [51]. Although no evidence at present to prove the direct regulatory relationship between PRL and MAPK15, given that PRL is the upstream pathway of the classical MAPK signal, we hypothesize PRL (pituitary)/MAPK15 (hippocampus)/NRF2 (hippocampus) axis might be the potential molecular mechanism by which *L. rhamnosus* KF7 exerts protective effects in D-galactose induced aging mouse model.

3.5. Protective effect of *L. rhamnosus* KF7 is associated with the production of ILA

Metabolites derived from the gut microbiota play a significant role in the pathogenesis of AD [52]. Therefore, we initially characterized the untargeted fecal metabolome in mice from both Mod and KF7 group. PCA and OPLS-DA demonstrated that there were significant differences in the composition of metabolites between the two groups (**Figure 5A-B**). A total of 57 significantly up-regulated DEMs and 78 down-regulated DEMs were identified (**Figure 5C**). These DEMs were mainly lipids and lipid-like molecules (27.41%), organic acids and derivatives (20.74%), phenylpropanoids and polyketides (12.59%), organoheterocyclic compounds (12.59%), etc (**Figure 5D**). Through ORA, we further identified the association of these DEMs with “Tryptophan catabolism” and “Tryptophan metabolism” (**Figure 5E**). Tryptophan is an essential amino acid critical for protein synthesis in humans as well as a key player in the microbiota-gut-brain axis [53]. The disruption of the metabolic homeostasis of tryptophan can cause harm to the body's cognition by deteriorating the gut-brain axis [54]. Heatmap analysis of the top 40 up-regulated DEMs revealed the presence of L-Tryptophan and ILA, which were also identified by LC-MS analysis (**Figure 5F**). We then analyzed correlations between these DEMs and the corresponding behavioral indicators of mice (Recognition index, Time spend in novel object, and Time spend in target quadrant) (**Figure 5G**). The PS(22:2(13Z,16Z)/16:0), xanthoxylol, L-phenylalanine, and ILA exhibited highly significant correlations with all three behavioral indicators ($P < 0.001$).

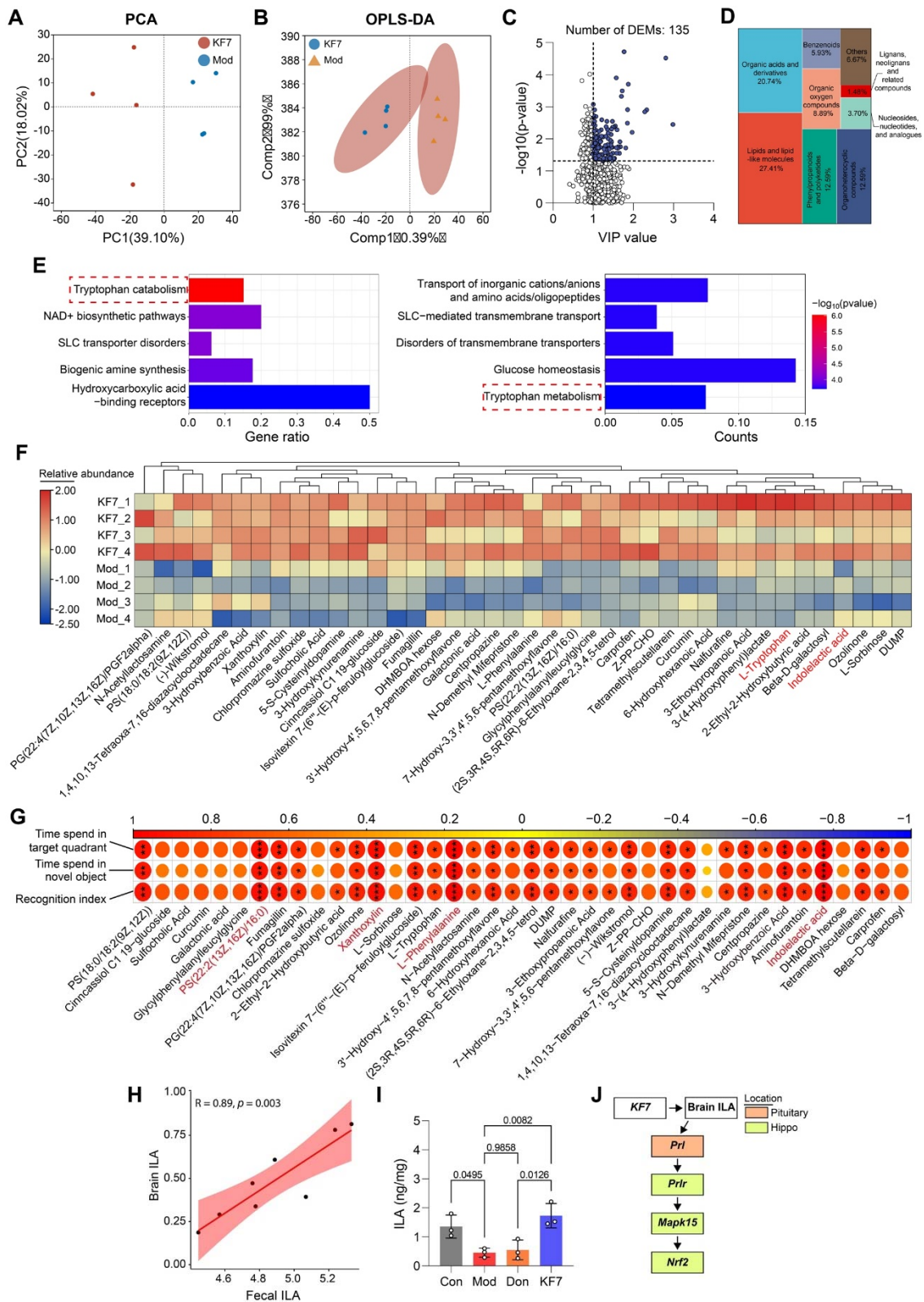


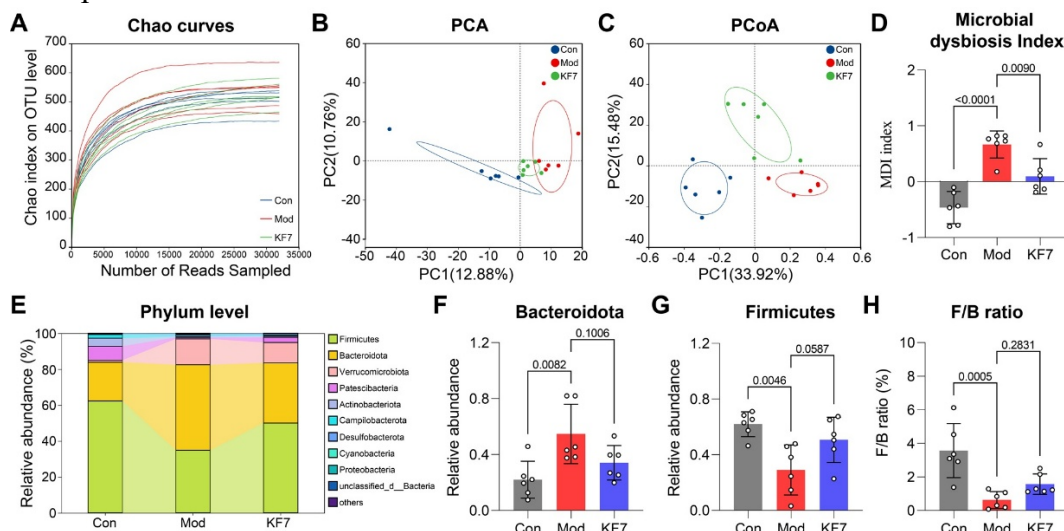
Figure 5. The effect of *L. rhamnosus* KF7 supplementation on the intestinal metabolic profile of D-galactose-induced aging mice. (A) PCA and (B) OPLS-DA of fecal metabolomes of mice from Mod and KF7 groups. (C) Minus-versus-add (MA) plot of DEMs. (D) The top 9 categories with the largest number of DEMs. (E) KEGG enrichment analysis of DEMs. (F) Heatmap analysis of the top 40 up-regulated DEMs. The metabolites in red font were also detected in the LC-MS analysis of neurotransmitters. (G) Correlation analysis of three behavioral indicators (Time spend in target quadrant (MWM), Time spend in novel object (NOR), and Recognition index (NOR)) and 40 up-regulated DEMs (n = 4 per group). (H) The level of ILA in brains of mice from Con, Mod, Don, and KF7 groups. (I) Correlation scatter plots of cereal ILA and fecal ILA levels. (J) The refined molecular mechanism underlying *L. rhamnosus* KF7-mediated attenuation of cognitive impairment. The p -value is directly indicated on the bar charts (I). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with n = 3–4 per group.

Given that cerebral ILA levels correlated with behavioral improvement in mice, we focused on this metabolite for subsequent analysis. We established a correlation curve between the cerebral ILA and fecal ILA, which revealed a significant positive correlation between them (**Figure 5H**). The ELISA results demonstrated significantly reduced cerebral ILA levels of Mod group mice compared to controls (**Figure 5I**). Supplement of *L. rhamnosus* KF7 significantly restored this trend ($P < 0.05$), whereas Donepezil showed no comparable restorative effect ($P > 0.05$). Based on these results, we revised the underlying molecular mechanism by which KF7 exerted its effect (**Figure 5J**). ILA has been shown to cross the blood-brain barrier (BBB) [55]. *B. breve*-derived ILA could cross the BBB and activate the AhR receptor, thereby exerting an antidepressant effect [56]. In another study, microbiome-derived ILA was shown to regulate microglia and astrocytes and alleviate neuroinflammation [57]. Besides, *in vitro* evidence implied ILA could stimulate the MAPK pathway in neurons [58] and the neuroprotective effect of ILA was related to the NRF2 pathway [59].

3.6. *L. rhamnosus* KF7 exerted its protective effect by enhancing the abundance of lactobacilli producing ILA in gut microbiota

The gut microbiota, which plays an important role in health and disease in humans, can be regulated by diet [60]. We then analyzed the impact of *L. rhamnosus* KF7 on the gut microbiota in D-galactose-treated mice and identified the key microbe that mediate the production of ILA.

The Chao curve of 16S rRNA gene sequences remained almost constant after a rapid increase (**Figure 6A**). This indicates that the current sequencing depth is sufficient to capture the vast majority of detectable OTUs in the sample. According to the β -diversity analysis, the OTUs varied among the Con, Mod, and KF7 groups according to the PCA and PCoA analyses (**Figure 6B-C**). This indicated that *L. rhamnosus* KF7 significantly altered the gut microbiota composition of D-galactose-treated mice. MDI quantifies the severity of microbial disruption, with higher values representing more pronounced imbalance [61]. D-galactose significantly increased the MDI index, while after the administration of KF7, the MDI was significantly reduced ($P < 0.05$, **Figure 6D**). MDI was significantly higher in the AD patients compared to the healthy control [62]. Our experimental results also confirmed this conclusion.



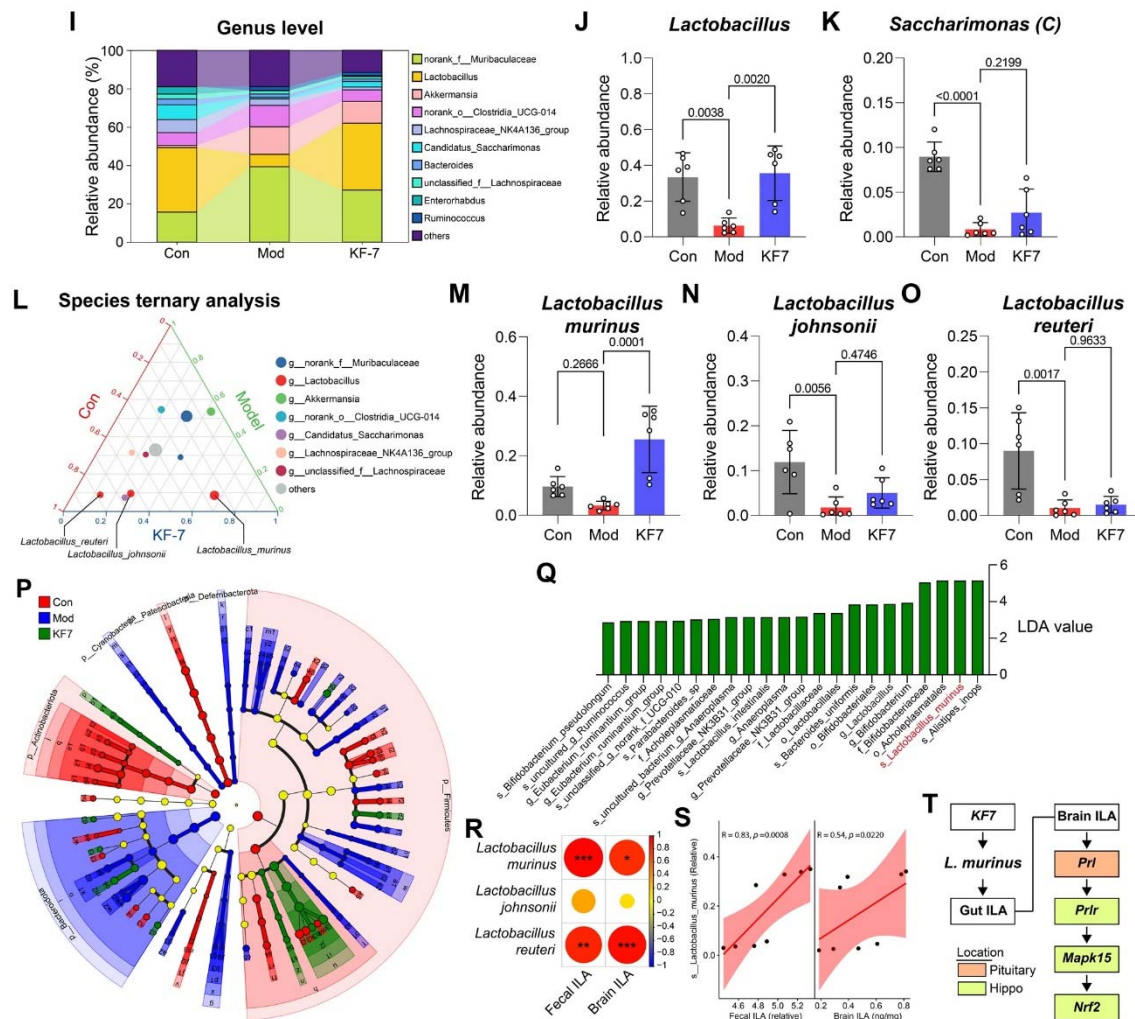


Figure 6. *L. rhamnosus* KF7 regulated the gut microbiota in D-galactose-induced aging mice. (A) Chao1 rarefaction curve of fecal 16S rRNA sequencing of mice from Con, Mod, and KF7 groups. (B) PCA and (C) OPLS-DA of the fecal 16S rRNA sequencing from three groups. (D) MDI analysis of fecal 16S rRNA sequencing from three groups. (E) Phylum-level species relative abundance analysis of three groups. Alterations in the relative abundance of (F) Bacteroidota, (G) Firmicutes, and (H) F/B ratio in three groups. (I) Genus-level species relative abundance analysis of three groups. Alterations in the relative abundance of (J) *Lactobacillus* and (K) *Candidatus-Saccharimonas* (*Saccharimonas* (C)) in three groups. (L) Species ternary analysis of three groups. Alterations in the relative abundance of (M) *L. murinus*, (N) *L. johnsonii*, and (O) *L. reuteri* in three groups. (P) Evolutionary branching diagram for LEfSe analysis. The Annotations of points can be found in **Supplemental Figure 4**. (Q) Histogram of the predominant gut microbiota in KF7 group based on LDA value from LEfSe analysis. (R) Correlation analysis of the relative abundances of bacteriums (*L. murinus*, *L. johnsonii*, and *L. reuteri*) and cereal/fecal ILA levels. (S) Correlation scatter plots of *L. murinus* and cereal/fecal ILA levels. (T) The MGBA underlying *L. rhamnosus* KF7-mediated attenuation of cognitive impairment. The *p*-value is labeled on the bar charts (I). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with *n* = 6 per group.

At the phylum level, Firmicutes, Bacteroidetes, Verrucomicrobiota, and Patescibacteria dominated the microbial community (**Figure 6E**). Compared with the Con group, the relative abundance of Bacteroidetes increased in the Mod group, whereas the abundance of Firmicutes decreased (**Figure 6F-G**). The F/B ratio significantly declined in the Mod group ($P < 0.05$) but increased in the KF7 group (**Figure 6H**). The decrease in relative abundance of Firmicutes and increase in relative abundance of Bacteroidetes have been observed in the elderly (70–85 years) compared to young adults [63]. At the genus level, *norank_f_Muribaculaceae*, *Lactobacillus*, and *Akkermansia* dominated the microbial community (**Figure 6I**). Treatment with *L. rhamnosus* KF7 reversed the D-galactose-induced reduction in the abundance of the

Lactobacillus and *Saccharimonas* (C) with a significant effect on *Lactobacillus* ($P < 0.05$, **Figure 6J-K**). The species trinity analysis showed three species, *L. murinus*, *L. johnsonii*, and *L. reuteri* exhibiting significantly higher abundance in both the Con and KF7 groups (**Figure 6L**). Treatment with *L. rhamnosus* KF7 reversed the D-galactose-induced reduction in the abundance of these species with a significant increment on *L. murinus* ($P < 0.05$, **Figure 6M-O**). In patients with AD, the abundance of *Lactobacillus* is significantly reduced ($P < 0.05$) [64]. Studies have shown that *Lactobacillus* can alleviate neuroinflammation and oxidative stress through varied mechanisms, thereby exerting protective effects on neurodegenerative diseases [65]. Supplementation of *Lactobacillus* has shown the potential to alleviate cognitive impairment [66].

LEfSe analysis was further performed to estimate the dominant bacteria in KF7 groups (**Figure 6P**). Among the top 10 LDA score taxonomies, Lactobacillales, Lactobacillaceae, *Lactobacillus*, *L. murinus* functioned in the KF7 group but differed from the other groups (**Figure 6Q**). The correlation analysis revealed significant positive associations between *L. murinus* and *L. reuteri* and fecal/ cerebral ILA levels ($P < 0.05$, **Figure 6R**). The correlation coefficients for *L. murinus* and Fecal/cerebral ILA levels were 0.83 and 0.54, respectively (**Figure 6S**). **Figure 6T** illustrated the MGBA of the *L. rhamnosus* KF7 improving D-galactose-induced aging model. In mice with Intestinal ischemia/reperfusion injury, *L. murinus* exhibited a beneficial effect on gastrointestinal disorders [67]. Administration of *L. murinus* reportedly alleviated inflammation and neuronal apoptosis in the CNS [68]. *L. murinus* has been proven to produce ILA in the gut and play a positive role in human health through regulating NRF2 [59]. In this study, we confirmed that the *L. rhamnosus* KF7 intervention could affect the abundance of *L. murinus* and the ILA level in the D-galactose-induced aging model mice. We also discovered that after the *L. rhamnosus* KF7 intervention resulted in the activation of NRF2 in the mice brain. Collectively, these data supported the hypothesis that *L. rhamnosus* KF7 intervention significantly increased the intestinal abundance of *L. murinus* in D-galactose-induced aging mice, leading to elevated concentrations of its metabolite ILA in the gut. ILA subsequently crossed BBB and then activated prolactin signaling cascades. This activation ultimately promoted nuclear translocation of NRF2 and ameliorated neuroinflammation and oxidative stress.

3.7. *L. rhamnosus* KF7 ameliorated intestinal metabolic dysregulation and enhanced lactic acid synthesis by the gut microbiota.

The gut microbiota is closely associated with the immunity and metabolic types in the host [69]. The functional prediction of the 16S sequence revealed that *L. rhamnosus* KF7 administration significantly ameliorated intestinal metabolic dysregulation, including Tryptophan and Glutathione metabolism ($P < 0.05$, **Figure 7**). Metabolites of tryptophan from gut microbial proteolysis are important for host health [70]. Gut microbial species can produce a variety of tryptophan catabolites, including ILA, which could improve cognitive impairment in D-galactose-induced aging mice [71]. This further supports the hypothesis we proposed. In addition, *L. rhamnosus* KF7 administration caused significant decrease in the abundances of gut microbiota involved in LPS synthesis and quorum sensing (QS) in D-galactose-induced aging mice. LPS produced by the gut microbiota could stimulate brain-resident immune cells, triggering neuroinflammation

and oxidative stress in brain [72]. A study reported that reduced LPS levels in the hippocampus in association with the improvement of D-galactose-induced cognitive impairment [73]. While no studies directly linked QS peptides to cognition, research demonstrated that gut microbiota-derived QS peptides maintained host intracellular homeostasis [74]. Various QS peptides have been demonstrated to cross the BBB, thereby exhibiting potential neuroregulatory effects [75].

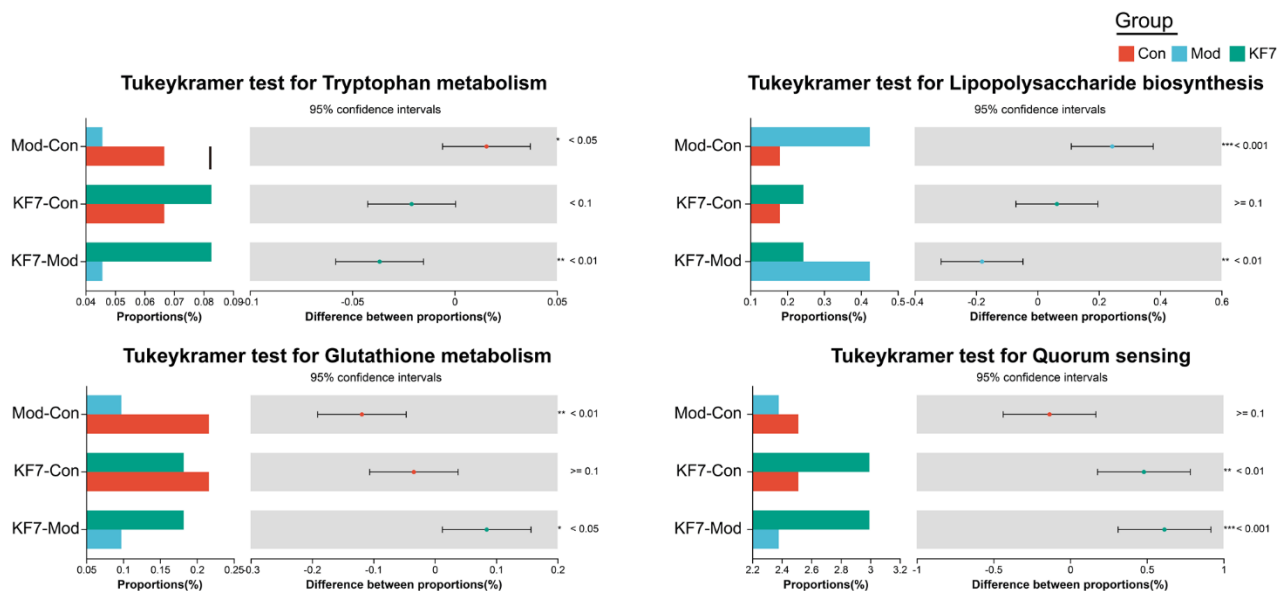


Figure 7. Functional prediction analysis of gut microbiota in Con, Mod, and KF7 groups based on KEGG pathways. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with $n = 6$ per group.

This study demonstrated the improvement effect of *L. rhamnosus* KF7 on cognitive impairment induced by D-galactose and the mechanisms. It also showed the potential of *L. rhamnosus* KF7 in improving AD-related phenotypes. However, further validation of the therapeutic effects of *L. rhamnosus* KF7 on AD is still required across additional AD models including scopolamine-induced models and APP/PS1 model.

4. Conclusions

In summary, this study demonstrated that *L. rhamnosus* KF7 exhibits significant potential in preventing D-galactose-induced cognition impairments in senescence mice. *L. rhamnosus* KF7 alleviated D-galactose-induced decreases of memory and learning ability, oxidative stress in the central nervous system, and neurotransmitter imbalance, and its distinct effects on the structure of gut microbiota unraveled the crucial role of gut microbiota in mediating therapeutic outcomes. Additionally, *L. rhamnosus* KF7 exerted its protective effects by activating the prolactin signaling pathway, thereby up-regulating NRF2 expression in brain. Our results suggest that gut microbiota-derived ILA is the key signal molecule that mediates the prevention of D-galactose-induced cognitive impairment by *L. rhamnosus* KF7 through MGBA. These mechanistic insights position *L. rhamnosus* KF7 as a promising nutraceutical candidate for preventing and ameliorating senescence related cognitive impairment. Our results provide theoretical support to develop a probiotics-based strategy for treating and preventing cognitive impairment and other age-related mental health issues.

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Declaration of competing interest

All the authors declare no conflict of interest.

Data availability

The data of RNA-seq, metabolome, and 16S sequencing have been submitted to the GenBank Sequence Read Archive (SRA) under accession number PRJNA1327054.

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