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# Exerkine irisin mitigates cognitive impairment by suppressing gut-brain axis-mediated inflammation

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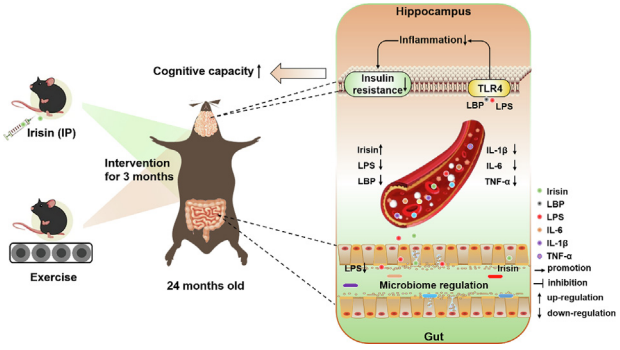
## HIGHLIGHTS

- Irisin can improve learning and memory capacity of aged mice.
- Irisin can reverse disturbed gut microbiota in aged mice.
- Irisin can optimize metabolites of gut microbiota in aged mice.
- Exercise-induced irisin as a mediator regulating gut microbiota and metabolites for suppressing gut-brain axis-mediated inflammation.

## GRAPHICAL ABSTRACT

Exercise-induced irisin ameliorates cognitive impairment of aged mice through rescuing the imbalanced gut microbiota and suppressing TLR4/MyD88 signal pathway-mediated insulin resistance.

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## ABSTRACT

*Introduction:* Exercise has been recognized to improve cognitive performance by optimizing gut flora and up-regulating exerkine irisin.
*Objective:* Although exercise-induced irisin is beneficial to cognitive improvement, whether this benefit is achieved by optimizing gut microbiota and metabolites is not fully explored.
*Methods:* After aerobic exercise and exogenous irisin interventions for 12 weeks, the 16S rRNA and metabolites in feces of 21-month-old mice were analyzed. Meanwhile, the differential miRNAs and mRNAs in hippocampal tissues were screened by high-throughput sequencing. Relevant mRNAs and proteins were evaluated by RT-PCR, Western blot, and immunofluorescence.
*Results:* Compared with the young control mice, irisin levels and cognitive capacity of aged mice revealed a significant reduction, while aerobic exercise and intraperitoneal injection of exogenous irisin reversed aging-induced cognitive impairment. Similarly, 147 up-regulated and 173 down-regulated metabolites were detected in aged mice, while 64 and 45 up-regulated and 225 and 187 down-regulated metabolites were detected in aged mice with exercise and irisin interventions, respectively. Moreover, during

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hippocampal miRNA and mRNA sequencing analysis, 9 differential gut flora and 35 differential genes were identified to be correlated with the inflammatory signaling mediated by the TLR4/MyD88 signal pathway.

*Conclusion:* Aging-induced cognitive impairment is due to insulin resistance induced by TLR4/MyD88 signaling activation in hippocampal tissues mediated by gut microbiota and metabolite changes. Myokine irisin may be an important mediator in optimizing gut microbiota and metabolism for an improved understanding of mitigated aging process upon exercise interventions.



## Introduction

Cognitive decline, as an important manifestation of aging, is common in neurodegenerative diseases such as Alzheimer's disease (AD), with cognitive impairment [1,2]. The global population aging reveals a rapid expansion with increasing number of the elderly suffering from cognitive decline. With the in-depth studies, more and more factors including aging, increased inflammation [3], impaired autophagy [4], excessive endoplasmic reticulum (ER) stress [5], abnormal circadian rhythm [6], and imbalanced gut microbiota [7] are involved in cognitive decline. Exercise as a non-pharmacological intervention can promote body health involving complex molecular mechanisms. It is worth noting that imbalanced gut microbiota can be induced by aging process [8] and the mitigation of cognitive decline through optimizing gut microbiota and their metabolites has been verified [9], which could become one of the key interventional targets for repealing global population aging [10].

Recent studies have confirmed that exercise-induced myokine irisin can enhance cognitive and memory capacity of aged mice; however, underlying mechanisms for alleviating impaired cognition through exercise-induced irisin-mediated optimization of gut microbiota and metabolites have less reported [11]. Irisin has been found to be involved in the regulation of intestinal microbiota in myocardial ischemia and colitis protection, and an obvious depression and a significantly cognitive decline have been observed in animal models with irisin deficiency [11–13]. Similarly, another study has demonstrated that irisin may exert anti-inflammatory effects by targeting the TLR4/MyD88 signal pathway [14]. As we all know, TLR4 is mainly activated by lipopolysaccharide (LPS) in the intestine, thereby impairing the balance of gut flora in the body. Coincidentally, during the aging process, the gut flora could be imbalanced, thereby resulting in the increased intestinal epithelial permeability, leading to an increased LPS level in the body, stimulating inflammatory factors, aggravating central nervous system inflammation, and triggering insulin resistance and cognitive impairment [15–17]. In a model of type 1 diabetes, injection of exogenous irisin reveals a significant reduction of intestinal epithelial permeability [18]. Relevant studies have also confirmed that cognitive decline of aging mice is closely related to the activation of TLR4 [19], and the TLR4 knockout mouse model during aging process shows improved brain structure and cognitive capacity [20]. Therefore, it may be of great significance to target the TLR4 signal pathway for the prevention and treatment of aging-induced cognitive impairment. Similarly, we hypothesize that exercise-induced myokine irisin is likely to be involved in the regulation of intestinal microorganisms to indirectly enhance the cognitive capacity of aged mice. Through high-throughput sequencing of gut microbiota and metabolites in feces and the screening and identification of differential miRNAs and mRNAs in hippocampal tissues from aged mice followed by exercise and irisin interventions, we could deeply uncover exercise-induced irisin for rescuing aging-induced cognitive impairment as a novel interventional strategy.

## Materials and methods

### *Recombinant irisin expression and purification*

The DNA sequence encoding irisin was cloned into pET-28a vector (MilliporeSigma, CA, USA) to obtain the pET-28a-irisin plasmid. The resultant pET-28a-irisin plasmid was then transformed into *Escherichia coli* BL21(DE3) competent cells, and spread on a Luria-Bertani (LB) agar plate containing kanamycin (50 μg/L) for incubation overnight at 37 °C. A single colony was picked up to inoculate into the LB medium containing kanamycin (50 μg/L) for cell culture with 200-RPM shaking at 37 °C. Upon the optical density at 600 nm of 0.6 for cell culture, isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) was added in cell culture for inducing the expression of histidine-tagged irisin during cell cultivation for another 8 h. To purify irisin, cell pellets were sonicated to disrupt cell membrane. The supernatant was filtered and injected into a nick-chelating agarose column (MilliporeSigma, CA, USA) loaded with 0.1 M nickel sulfate solution. The bound irisin protein was eluted with a gradient of imidazole from 10-500 mM in phosphate buffer. The collected protein was dialyzed in 500 mM Tris buffer (Servicebio Technology, Wuhan, China) to remove imidazole and endotoxin through dialysis membrane with the cutoff of 10000 Da. The concentration of purified recombinant irisin was determined by the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and used for the intervention of animal experiments later.

### *Animal grouping and interventions*

Thirty 4-month-old specific pathogen-free (SPF) grade male C57BL/6 mice were fed with adequate maintenance feed and water until 21 months old, and then randomly divided the aged mice into a non-intervention group (OC), an aerobic exercise group (OE), and an irisin intraperitoneal injection group (OI), with 10 mice in each group; and ten 4-month-old mice were used as the young control group (YC). Furthermore, room temperature (25 ± 2 °C), relative humidity (50–60%), circadian rhythm (12 h:12 h), and free accessibility of foods and water were kept at same for all mice. All mice in the OE group were subjected to running training on treadmill with a slope of 10° for 12 weeks. At the beginning of running training, the mice were provided with adaptive treadmill running for 5 days (start with 4.2 m/min until 12 m/min, with an increasing interval of 1 m/min every 30 s), with exercise training time of 10, 20, 30, 45 and 45 min in first five days, respectively. After adaptive running training, the formal aerobic exercise training (12 m/min) with the training duration of 45 min and 5 times of training within one week was conducted for 12 consecutive weeks. In the OI group, the mice were provided with the intraperitoneal injection of exogenous recombinant irisin according to their body weights (500 μg/kg). Both aerobic exercise training and irisin injection were performed 5 times a week at the fixed time and the health status of all aging mice during the interventions was also recorded.

*Ethics statement*

All experiments involving animals were conducted according to the ethical policies and procedures approved by Institutional Animal Care and Use Committee of Wuhan Sports University with the certificate number of S087-21-05D, and complied with the internationally recognized 3R principles.

*Behavioral testing*

The learning and memory capacity and behavior of all mice were evaluated by Morris water maze (MWM) testing. On the 1st day, a positioning test was conducted, and the mice were released to four different locations in the east, west, south, and north directions, and allowed to freely explore and swim for 60 s to adapt to the environment. In the next 4 days, the mice were released for 4 times within one day at different starting points. If the mice did not find the platform within 60 s, they were guided to the platform and stayed on the platform for 10 s for spatial location memory. On the 5th day, after the platform was removed, the mice were released in the quadrant opposite the platform to freely swim for 60 s, and the time of targeting the platform quadrant and the number of crossing the platform of the mice were used as the indicators to evaluate the spatial learning and memory capacity.

*Blood sample collection and ELISA*

Prior to sample collection, all mice were fasted with free accessibility of water for 12 h, and sacrificed by carbon dioxide inhalation for collecting blood samples in sterile EP tubes from eyeballs, and the collected blood samples were stood quietly at room temperature overnight. On the next day, the blood samples were subjected to 20-min centrifugation at 3000×*g* and 4 °C, and the supernatant was harvested for corresponding analysis. Irisin in serum of the mice was determined by its ELISA kit (Phoenix Pharmaceuticals, California, USA), and LPS, IL-1β, IL-6, TNF-α and LBP in serum were evaluated by corresponding ELISA kits (Meimian Industrial Co., Ltd, Jiangsu, China) in accordance with the manufacturer's operation procedures.

*Collection and analysis of hippocampal tissue*

*Transmission electron microscope (TEM) inspection*

After completing MWM testing, the mouse was sacrificed by carbon dioxide inhalation, and the hippocampal tissue at 1 $mm^3$ was harvested, immediately fixed in 250 μL of 2.5% glutaraldehyde containing 0.1 M sodium cacodylate (pH 7.2), subsequently post-fixed in 1% osmium tetroxide for 1 h at 4 °C, stained with uranyl acetate, and then embedded with resin. The processed block of hippocampal tissue was sectioned into 70 nm ultra-thin sections. The ultrastructure of hippocampal tissues was examined and photographed under a TEM (HT7700 Hitachi, Tokyo, Japan) at Medical and Structural Biology Research Center of Wuhan University.

*Histological analysis of hippocampal tissue*

Similarly, after MWM test was completed, 3 mice from each group were randomly selected on the next day, and then subjected to the perfusion using saline and 4% paraformaldehyde (pH 7.4) in the anesthesia status. The perfused mice were sacrificed by carbon dioxide inhalation, the whole brains were harvested to fix in 4% paraformaldehyde overnight, sequentially embedded in paraffin, and cut into the slices in the thickness of 5 μm using a cryotome. After the paraffin removal, the tissue slices were subjected to hematoxylin-eosin (HE), Nissl, and immunohistochemical staining, respectively. The histological images of hippocampal tissues were acquired by imaging system (Eclipse-E100, Nikon, Japan) and analyzed by ImageJ Pro software (NIH, Bethesda, MD, USA).

*Sequencing of miRNAs and mRNAs in hippocampal tissue*

According to the operation procedures of the Trizol extraction kit (Sangon Biotech, Shanghai, China) and Qubit2.0 RNA detection kit (ThermoFisher, MA, USA), total RNA in hippocampal tissue was extracted and quantitatively determined. The integrity and contamination of RNA were evaluated by agarose gel.

miRNA: Total miRNA was extracted and processed for sequencing by Illumina NovaSeq6000 platform (Illumina, TX, USA). FastQC (v0.11.2) was utilized to evaluate the quality of sequencing data for all samples, the Adapter was removed and the quality was controlled by Trimmomatic. Bowtie2 (v2.2.4) software was applied for mapping the clean data to the reference genome. The miRDeep2 (v2.0.0.8) software and miRbase (v22.1) database were used to match and identify miRNAs.

mRNA: Total mRNA was extracted and processed for sequencing by Illumina NovaSeq6000 platform (Illumina, TX, USA). FastQC (v0.11.2) was used for evaluating the quality of sequencing data for all samples, the Adapter was removed and the quality was controlled by Trimmomatic (v0.36). Qualimap (v2.2.1) was used to check the distribution of uniformity and genome structure according to comparison results for further analysis. StringTie (v1.3.3b) was applied to construct known gene models for evaluating gene expression levels. Co-expression analysis of genes was conducted by WGCNA (v1.51), and multi-directional statistical analysis was performed based on sample expression matrix for sample comparison analysis. The R package DESeq (v1.26.0) was used for the analysis of the difference in gene expression, and the graph visualization was completed based on ggplot2 (v2.2.1). The interaction network between miRNAs and genes was constructed by using the igraph R package (v1.0.1) association analysis.

Dimensionality reduction analysis was implemented based on principal component analysis (PCA), the volcano plot was established to visualize differential expression, and the enrichment bubble map was generated to visualize the KEGG pathway enrichment analysis. The data in this study are mainly analyzed with the help of NCBI NR, NCBI NT, KEGG and other databases.

*The analysis of 16S rRNA sequencing and gut microbiota in feces*

After experimental interventions, fresh feces of the mice from all groups were collected for extracting DNA by magnetic soil and stool DNA kit (Sangon Biotech, Shanghai, China). The purity of the extracted DNA was validated by agarose gel electrophoresis. The 16S rRNA gene was amplified by PCR and purified by 2% agarose gel electrophoresis. The target band was recovered by the Universal DNA Purification and Recovery Kit (ThermoFisher, MA, USA). NEB Next® Ultra DNA Library Prep Kit (Bio-Rad Laboratories, San Francisco, USA) was used for the construction of gene library. After the library was qualified, the V3-V4 region of 16S rRNA was sequenced using the NovaSeq6000 sequencing platform (Illumina, TX, USA). Quality of raw data was evaluated by FastQC (v0.11.2), Trimmomatic (v0.36) was used for removing adapter and quality control, and Flash (v1.2.7) was used for splice data to obtain clean data. Qiime (v1.9.1) was used to denoise, cluster, and annotate with species. Then, α-diversity and β-diversity analysis (Permanova test), cluster analysis (PCA analysis), difference analysis (LEfSe), and correlation analysis of the species were carried out at each taxonomic level.

*Sequencing of non-targeted metabolites in gut*

Fecal samples were thawed on ice after being removed from the −80 °C freezer, and approximately 20 mg of the samples were weighed for metabolite extraction. Non-targeted metabolites were collected using ultra-high performance liquid chromatography

(UPLC) (SCIEX, MA, USA) and quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) (TripleTOF6600, AB SCIEX, MA, USA) for data acquisition. Extensive targeted detection data were acquired by UPLC (SCIEX, MA, USA) and tandem mass spectrometry (MS/MS) (SCIEX, MA, USA). The data was processed by Progenesis QI software (v3.0), and the data was imported into EZinfo3.0 (v3.0.3) software for multidimensional statistical analysis. Metabolites were analyzed by PCA (R-Base Package v3.5.1) and orthogonal partial least squares discriminant analysis (OPLS-DA) (MetaboAnalystR v1.0.1) for multidimensional statistical analysis between two groups. The differential metabolites with vasoactive intestinal peptide (VIP) > 1 were screened and subjected to unidimensional statistical analysis (Welch's *t*-test and fold change analysis). Moreover, differential metabolites meeting the criteria (VIP > 1, $p < 0.05$ and fold change > 2) were screened and subjected to final compound identification analysis. The HMDB and METLIN databases were searched based on the primary and secondary spectra to obtain the identification results of differential metabolites. MetaboAnalyst (v5.0) online software was used to analyze the signal pathway and enrichment of differential metabolites.

*qRT-PCR*

RNA-easy isolation reagent was used for extracting mRNA in hippocampal tissues. HiSipt@ II Q RT Supermix (Vazyme, Nanjing, China) was used for qRT-PCR to reverse-transcribe mRNA into cDNA after removing genomic DNA. Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) was used for qRT-PCR of mRNA. The primers were synthesized from Sangon Biotech (Table 1). Quant Studio 1 Real-Time PCR System (ThermoFisher, MA, USA) was used to perform qRT-PCR with 20 μL reaction system for each sample in triple repeats using β-actin as the control, and the $2^{-\triangle\triangle CT}$ method was performed for all calculations.

*Western blot*

Hippocampal tissue samples from the mice were collected and immediately frozen in liquid nitrogen, and then stored in −80 °C refrigerator for future evaluation. During western blot analysis, frozen hippocampal tissue samples were taken out and homogenized in lysis buffer supplemented with protease and phosphatase inhibitors (Servicebio Biotechnology, Wuhan, China), and then extensively lysed for 30 min on ice. The lysed samples were centrifuged at 10,000× g for 5 min at 4 °C to obtain supernatants and the concentrations of total protein in supernatants were measured by a BCA kit (Beyotime Biotechnology, Shanghai, China). An aliquot of the supernatant was mixed with reduced protein loading buffer and heated at 95 °C water bath for 5 min to denature proteins. Approximately 25 μg of total protein in prepared samples were separated by using 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, MA, USA). The target protein was probed by primary antibodies against JNK, p-JNK, IL-1β, IL-6, TNF-α, TLR4, MyD88, MKK7, p-MKK7, p38 MAPK, p-p38 MAPK, IRS, and p-IRS, as well as corresponding secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). The band of the target protein after incubated with enhanced chemiluminescence (ECL) (Bio-Rad Laboratories, San Francisco, USA) reagent was imaged and visualized by an ultra-sensitive fluorescence/chemiluminescence imaging system, ChemiScope6300 (CLiNX Science Instruments, Shanghai, China).

**Table 1**
Primer information for qRT-PCR.

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| LBP | GGCTTGGCGTGGTCACTAA | TGCCGACTTTGGATTCGATCA |
| TLR4 | AGATCTGAGCTTCAACCCCT | TGTTTCAATTTCACACCTGGATAA |
| MyD88 | CTCGCAGTTTGTTGGATGCC | TTTCTGGCAGTCCTCCTCGAT |
| MKK7 | CGCGTCCTGGTTTAAGGATGT | CTCCAGACTCCCACTGAAGAA |
| JNK | GAACAGGATTGAGTAGCGGC | ATATTCACCAAGGCCGGCAG |
| p38 MAPK | AAAGGACCTACCGAGAGTTGC | GTCACCAGGTACACGTCATT |
| NF-κB | CCGAACTTCTCGGACAGCTT | GTTCGAGTAGCCATACCCTGG |
| TNF-α | CCAAATGGCCTCCCTCTCAT | CCACTTGGTGGTTTGCTACGA |
| IL-1β | GCTTCCTTGTGCAAGTGTCT | TGACCACTCTCCAGTACCCA |
| IL-6 | GACTGGGGATGTCTGTAGCTC | CAACTGGATGGAAGTCTCTTGC |

*Statistical analysis*

All above experiments were accomplished according to the designated procedures (Fig. 1) and all collected data were presented as mean ± standard deviation (M ± SD) to conduct the statistical analysis by GraphPad Prism software (v4.0.2). Two-tailed unpaired student's t-test and the Wilcoxon sum test were utilized to evaluate the statistical significance between two or multiple groups, respectively. Statistical analysis among multiple groups was implemented based on Kruskal-Wallis test for non-parametric data, and the significant difference was considered at $p < 0.05$. Spearman correlation analysis was used for correlation analysis.

## Results

*Exercise and irisin interventions reversed aging-induced reduction of cognitive capacity in mice*

The aging-induced reduction of cognitive capacity can be reversed by aerobic exercise [21], but whether it is correlated with exercise-induced myokine irisin is still unknown. Therefore, the 21-month-old mice were provided with aerobic exercise training and intraperitoneal injection of exogenous irisin for 12 weeks, and the irisin level in serum and cognitive capacity of the mice were evaluated. The irisin level in the OC group were decreased significantly ($p = 0.0154$) when compared with the YC group. Oppositely, compared with the OC group, the irisin in OE and OI groups exhibited an obvious increase ($p = 0.0134$, $p = 0.0037$). Notably, the irisin level in the OI group was higher than that in the OE group (Fig. 2a). Based on cognition evaluation of the mice through MWM testing, compared with the YC group, the escape latency to platform increased significantly ($p = 0.0213$), while the target quadrant-exploring time and the platform-crossing number of the mice from the OC group on the 5th day were markedly reduced ($p = 0.0067$, $p = 0.0021$); in contrast, the reduced escape latency to platform, and the increased target quadrant-exploring time and platform-crossing number of the mice from OE and OI groups on the 5th day were observed ($p = 0.0041$, $p = 0.0425$; $p = 0.0005$, $p = 0.0491$; $p = 0.0002$, $p = 0.0075$) (Fig. 2c-e). These results demonstrated that 24-month-old mice exhibited the significant down-regulation of irisin in serum and the reduced cognitive capacity, which could be reversed by aerobic exercise and intraperitoneal injection of exogenous irisin.

*Exercise and irisin interventions rescued aging-induced damage of hippocampal tissues in mice*

Aging process is accompanied by damaged morphology, dysfunction, and reduced neuron number of hippocampal tissues; in contrast, exercise can effectively reverse these changes, as confirmed by HE and Nissl staining and immunohistochemical analysis. Hippocampal tissues of the mice from the OC group had sparse neurons and unclear nucleoli and nuclei, as shown in darker image by Nissl staining (Fig. 3a and b), and significantly reduced the number of neurons in CA1, CA3 and DG areas when compared with the YC group ($p = 0.0015$, $p = 0.0076$, $p = 0.0009$) (Fig. 3c). On the

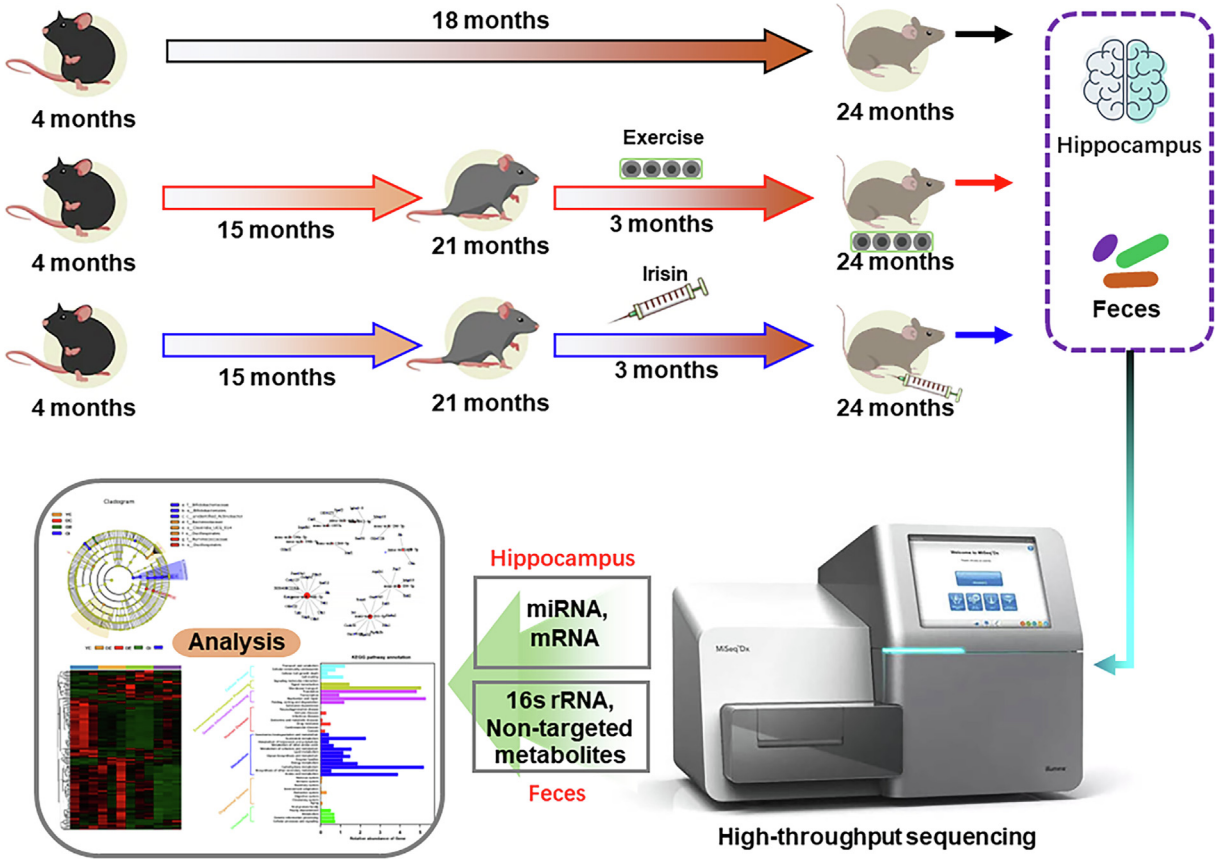



**Fig. 1.** Schematic diagram of the experimental process.

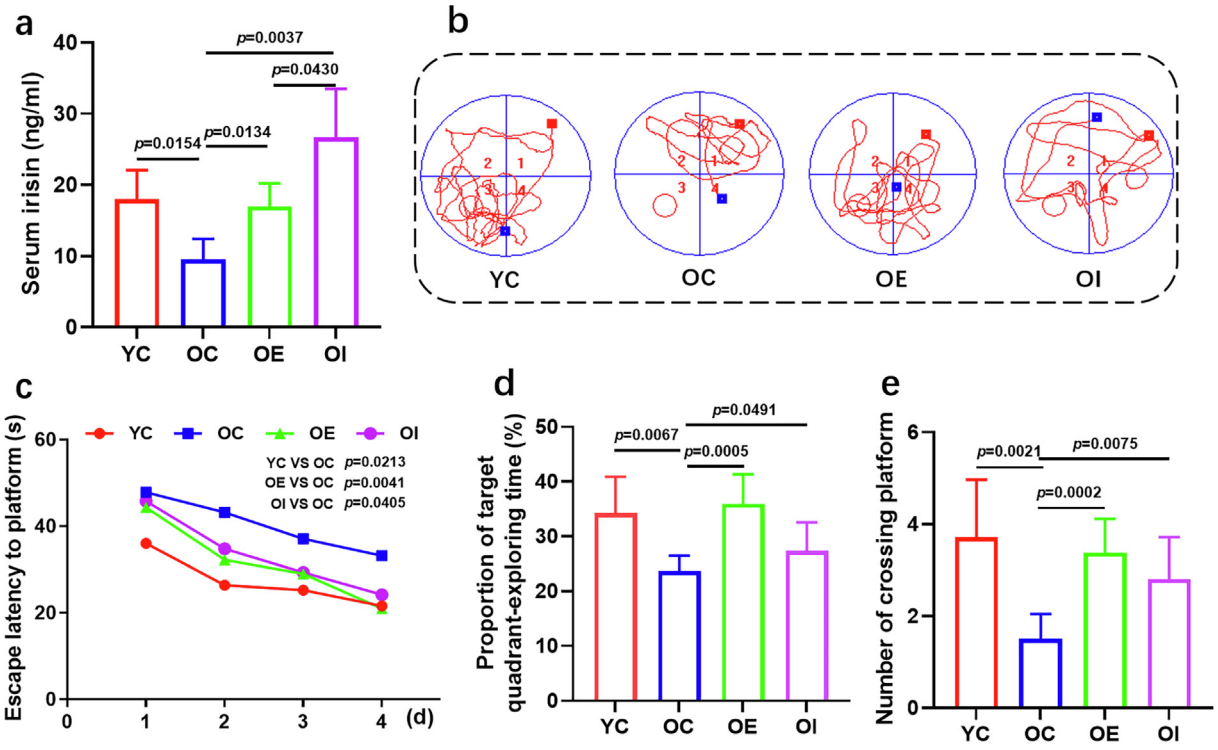



**Fig. 2.** Irisin levels in serum and cognitive capacity of the mice from different groups. (a) Irisin levels in serum of the mice from different groups; (b) Swimming track during MWM testing; (c) The escape latency to platform during MWM testing; (d) The proportion of target quadrant-exploring time of the mice on the fifth day; (e) The number of crossing platform of the mice on the fifth day.

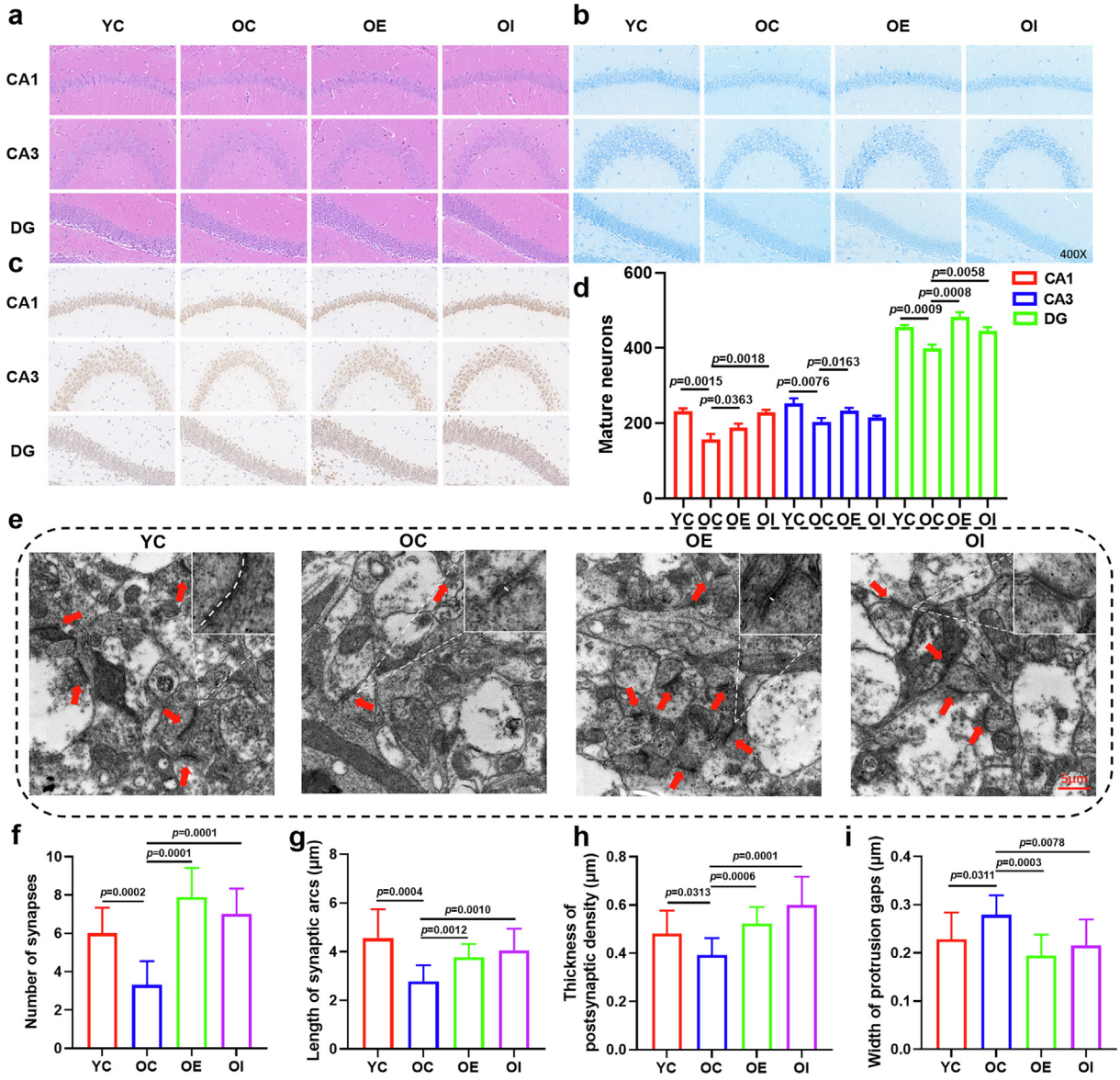



**Fig. 3.** HE and Nissl staining, and TEM observation of neurons in hippocampal tissues of the mice from each group. (a) HE staining and (b) Nissl staining of hippocampal tissues in the mice from different groups; (c) Immunohistochemical staining of mature neurons; (d) The number of mature neurons in different regions of hippocampal tissues; (e) TEM observation of hippocampal tissues; (f) The number of synapses; (g) The length of synaptic arcs; (h) The thickness of postsynaptic density; (i) The width of protrusion gaps. Red arrows indicate synapses.

contrary, compared with the OC group, the neurons in CA1, CA3 and DG areas of hippocampal tissues in mice from OE and OI groups revealed more ordered and dense arrangement, and less damage and more matured neurons ($p$ = 0.0363, $p$ = 0.0163, $p$ = 0.0008; $p$ = 0.0018, $p$ = 0.0058) (Fig. 3d). These results suggested that neurons in CA1, CA3 and DG areas in hippocampal tissues of aged mice were disordered and sparse during aging process, and the aging-induced reduction of neuronal function and number could be reversed by aerobic exercise and intraperitoneal injection of exogenous irisin.

Similarly, aging accelerated the loss of neuronal synapses, increased protrusion gaps, reduced synaptic arc lengths, and decreased postsynaptic density, the hallmarks of synaptic plasticity, as shown in TEM images (Fig. 3e). Indeed, based on the statistical analysis, the number of neuronal synapses decreased significantly ($p$ = 0.0002) (Fig. 3f), the length of synaptic arc and the thickness of postsynaptic density decreased ($p$ = 0.0004, $p$ = 0.0313) (Fig. 3g and h), and synaptic gaps increased in the OC group ($p$ = 0.0311) when compared with the YC group (Fig. 3i). On the other hand, compared with the OC group, hippocampal tissues of the mice from OE and OI groups revealed significantly increased number of neurons, extended length of synaptic arcs and thickness of postsynaptic density, and reduced synaptic cleft ($p$ = 0.0001, $p$ = 0.0012, $p$ = 0.0006, $p$ = 0.0003; $p$ = 0.0001, $p$ = 0.010, $p$ = 0.0001, $p$ = 0.0078). Therefore, aging-induced reduction in the number of synapses, the length of synaptic arc strings, and the thickness of postsynaptic density, as well as the increase in protrusion gaps in hippocampal neurons could be reversed by aerobic exercise and intraperitoneal injection of exogenous irisin.

### *Exercise and irisin interventions optimized gut microbiota and metabolites in feces of aged mice*

#### *Exercise and irisin interventions rescued imbalanced gut microbiota of aged mice*

Gut microbiota is an important mediator for reducing cognitive impairment during exercise [22], but whether exercise-induced irisin and optimized gut microbiota have the capability to suppress

cognitive impairment during aging process is still unknown. Therefore, in the present study, gut microbiota of aging-induced mice following exercise and irisin interventions were analyzed by 16S rRNA sequencing. Four animal experimental groups with 6 mice in each group, 1,527,506 original reads were obtained from a total of 24 samples, and 1,126,634 valid reads were obtained after quality control. After all samples were clustered according to 97% homology, 80,165 OTUs were obtained. The number of OTUs were 976, 640, 698 and 690 in YC, OC, OE, and OI groups, respectively (Fig. 4a), indicating aging-induced disturbance of gut microbiota, but exercise and irisin interventions can alleviate this disturbance. The species abundance at the top 10 phylum and genus levels showed that *Bacteroidetes*, *Firmicutes* and *Actinomycetes* were dominant at the phylum level (Fig. 4b), and *Ligilactobacillus* and *Lactobacillus Lachnospiraceae_NK4A136_group* were dominant at the genus level (Fig. 4c). The differential analysis of the top 10 species at the phylum and genus levels found that *Firmicutes/Bacteroidota* (F/B) ratio and *Actinobacteria* were significantly reduced in the OC group at the phylum level ($p$ = 0.0009, $p$ = 0.0004) when compared with the YC group (Fig. 4d and e); while significantly reversed this ratio and level in OI group ($p$ = 0.0302, $p$ = 0.00024), and the mice from the OE group with exercise intervention showed reversed *Actinobacteria* level ($p$ = 0.0094). Similarly, compared with the YC group, the level of *Unidentified_Bacteria* in the OC group was significantly increased ($p$ = 0.0238) (Fig. 4f), while the mice from the OE group exhibited a significantly reversed trend for this change ($p$ = 0.0262), and the OI group showed a reversal sign, but no significant difference. At the genus level, *Bacteroides*, *Turicibacter*, *Parabacteroides* and *Bifidobacterium* in the OC group were reduced ($p$ = 0.0017, $p$ = 0.0148, $p$ = 0.0004, $p$ = 0.0005), while the OE and OI groups present a reverse in the reduction of *Bacteroides, Parabacteroides* and *Bifidobacterium* ($p$ = 0.0357, $p$ = 0.0035; $p$ = 0.0181, $p$ = 0.0197; $p$ = 0.0094, $p$ = 0.0024) (Fig. 4g-i and Supplementary Fig. S1), and the OE group did not exhibit the decline of *Turicibacter* (Fig. 4h) when compared with the YC group, suggesting that exercise and irisin interventions should participate in optimizing the balance of gut microbiota during aging process.

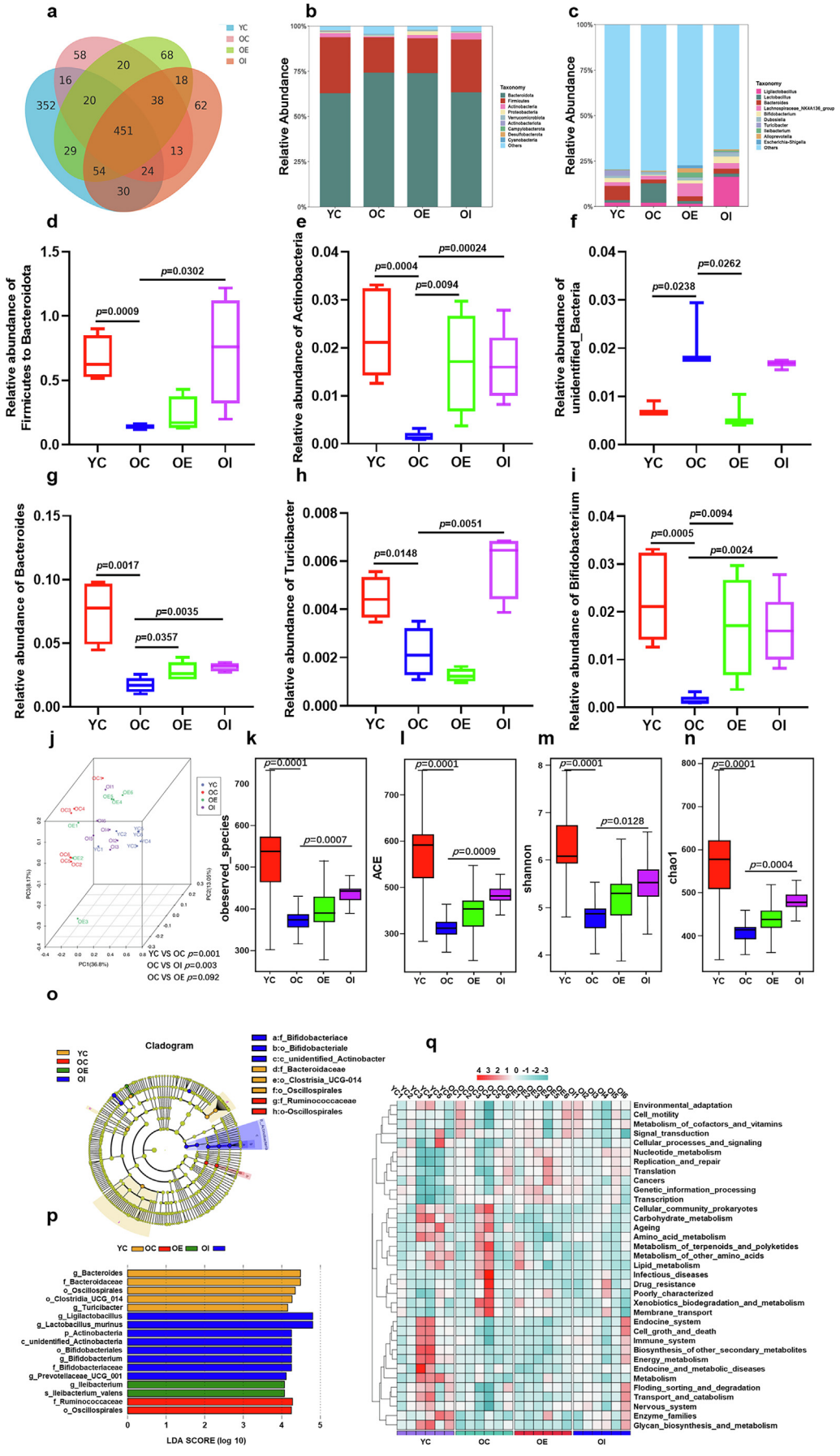
The principal coordinate analysis (PCoA) showed the distribution of bacterial community structure among different groups. ADONIS analysis showed the difference between YC and OC groups ($p$ = 0.001), and between OC and OI groups ($p$ = 0.003). However, the OC and OE groups have no difference ($p$ = 0.092) (Fig. 4j). The Good Coverage rate of each sample was above 99%, and the α-diversity analysis (Fig. 4k-n) of gut microbiota from each group was carried out through observed species, chao1, ACE and Shannon index. The observed species, chao1, ACE and Shannon in the OC group were reduced significantly ($p$ = 0.0001), and the mice from the OI group significantly reversed this trend ($p$ = 0.007, $p$ = 0.0009, $p$ = 0.0128, $p$ = 0.0004) when compared with the YC group. Although the mice from the OE group had a reversed trend, the statistical results were insignificant. These results suggested that intraperitoneal injection of irisin could reverse the change in the abundance and structure of gut microbiota caused by aging, thus contributing the compositions of gut microbiota like younger individuals, although exercise showed a particular improvement of recognitive capacity even without significant difference.

At the same time, potential biomarkers were screened through LEfSe analysis (LDA > 4) for differential microbiota in different groups (Fig. 4o). *Bacterodies*, *Bacterodaceae*, *Oscillospirales* and *Clostridia_UCG_014* were enriched in the YC group, *Ruminococcaceae* and *Oscillospirales* were enriched in the OC group, *Lleibacterium* and *Lleibacterium_valens* were enriched in the OE group, and *Ligilactobacillus*, *Lactobacillus_murinus*, *Actinobacteria*, *unidentified_Actinobacteria*, *Bifibobacteriales*, *Bifibobacterium*, *Bifibobacteriaceae* and *Prevotellaceae_UCG_001* were more abundant in the OI group. In addition, KEGG functional prediction and cluster analysis of gut microbiota based on Tax4Fun found (Fig. 4q) that the aging of the mice could cause the changes in the compositions of gut microbiota, which may be an important factor for inducing and exacerbating the disease. Aerobic exercise and intraperitoneal injection of exogenous irisin may rescue the imbalanced gut microbiota to alleviate aging, inflammation, and neurodegeneration-related diseases.

### *Exercise and irisin interventions regulated fecal non-targeted metabolites in aged mice*

Exercise in regulating fecal metabolites has been reported in many studies [23] and myokine irisin plays an active role in the regulation of gut microbiota. Therefore, quality control on fecal non-targeted metabolites was conducted. OPLS-DA analysis (Fig. 5a) showed that non-targeted metabolites could effectively distinguish the differential metabolites in feces of the mice from YC, OC, OE, and OI groups. In the statistical analysis of detected metabolites ($|\log_2 FC| \geq 1$, VIP $\geq 1$), compared with the YC group, 147 up-regulated and 173 down-regulated metabolites were detected in the OC group (Fig. 5b); compared with the OC group, 225 down-regulated and 64 up-regulated metabolites in the OE group were screened; similarly, 187 down-regulated and 45 up-regulated metabolites in the OI group were screened (Supplementary Fig. S2). Among them, compared with the YC group, 2-hydroxy-3,5-dinitro benzoic acid and 7-keto deoxycholic acid were decreased in mice from the OC group; while both exercise and irisin interventions could result in the significant increase of these compounds. Compared with the YC group, aging could induce the decrease of tetrahydro curcumin, while exercise intervention could reverse its decrease. Compared with the YC group, dihydrouracil and S-ribosyl-L-homocysteine in the OC group were reduced; while irisin intervention could rescue their reduction. Based on KEGG analysis, 94 pathways were enriched between YC and OC groups, 118 pathways were enriched between OC and OE groups, and 83 pathways were enriched between OC and OI groups. These pathways with significant differences were involved in serotonergic synapse ($p$ = 0.0197) and cAMP signaling pathway ($p$ = 0.0197) between YC and OC groups (Fig. 5c); pentose phosphate pathway ($p$ = 0.0052), neuroactive ligand-receptor interaction ($p$ = 0.0138), gap junction ($p$ = 0.0197), synaptic vesicle cycle ($p$ = 0.0206), taste transduction ($p$ = 0.0490) between OC and OE groups (Fig. 5d). Gonadotropin-releasing hormone (GnRH) secretion ($p$ = 0.0341) and cAMP signaling pathway ($p$ = 0.0463) were predicted between OC and OI groups (Fig. 5e), which may be related to nervous system function.

To further explore the effect of exercise and irisin interventions on fecal microbiome and metabolites, a correlation analysis was conducted by extracting top 30 differential metabolites and microorganisms (Fig. 5f and Supplementary Table S1). The statistical difference was observed with the reduction of *Bifidobacterium*, *Turicibacter*, *Parabacteroides* and *Bacteroides* in mice from the OC group at the genus level, partially reversed in OE and OI groups (Fig. 4g-i and Supplementary Fig. S1). Among them, *Bifidobacterium* was significantly positively correlated with 2-(2R)-2-methyl-2-pyrrolidinyl-1H-benzimidazole-7-carboxamide, and pantothenate ($p$ = 0.0231, $p$ = 0.0417). *Parabacteroides* was significantly positively correlated with cyclamic acid, 2-(2R)-2-methyl-2-pyrrolidinyl-1H-benzimidazole-7-carboxamide, pantothenate, L-phenylalanine, and dodecanedioic acid ($p$ = 0.0010, $p$ = 0.0345, $p$ = 0.0172, $p$ = 0.0351, $p$ = 0.0001). *Turicibacter* was significantly positively correlated with cyclamic acid ($p$ = 0.0009). *Bacteroides* was significantly negatively correlated with *sn*-glycero-3-phosphocholine, methylcysteine and heparin ($p$ = 0.0093, $p$ = 0.0212, $p$ = 0.0246), and positively correlated with cyclamic acid ($p$ = 0.0048). These results suggest that exercise and irisin-induced changes in gut microbiota may also affect the compositions of metabolites in


a
YC
OC
OE
OI
58
20
68
16
18
352
20
38
62
451
29
13
54
24
30
b
Relative Abundance
Taxonomy
Bacteroidota
Firmicutes
Actinobacteria
Proteobacteria
Verrucomicrobiota
Patescibacteria
Cyanobacteria
Desulfobacterota
Others
c
Relative Abundance
Taxonomy
Ligilactobacillus
Lactobacillus
Bacteroides
Lachnospiraceae_NK4A136_group
Bifidobacterium
Dubosiella
Turicibacter
Ileibacterium
Alloprevotella
Escherichia-Shigella
Others
d
Relative abundance of Firmicutes to Bacteroidota
p=0.0009
p=0.0302
e
Relative abundance of Actinobacteria
p=0.0004
p=0.0094
p=0.00024
f
Relative abundance of unidentified_Bacteria
p=0.0238
p=0.0262
g
Relative abundance of Bacteroides
p=0.0017
p=0.0357
p=0.0035
h
Relative abundance of Turicibacter
p=0.0148
p=0.0051
i
Relative abundance of Bifidobacterium
p=0.0005
p=0.0094
p=0.0024
YC
OC
OE
OI
j
PC1(36.8%)
PC2(13.95%)
PC3(8.17%)
YC VS OC p=0.001
OC VS OI p=0.003
OC VS OE p=0.092
k
obeserved_species
p=0.0001
p=0.0007
l
ACE
p=0.0001
p=0.0009
m
shannon
p=0.0001
p=0.0128
n
chao1
p=0.0001
p=0.0004
o
Cladogram
a:f_Bifidobacteriace
b:o_Bifidobacteriale
c:c_unidentified_Actinobacter
d:f_Bacteroidaceae
e:o_Clostrisia_UCG-014
f:o_Oscillospirales
g:f_Ruminococcaceae
h:o-Oscillospirales
p
g_Bacteroides
f_Bacteroidaceae
o_Oscillospirales
o_Clostridia_UCG_014
g_Turicibacter
g_Ligilactobacillus
g_Lactobacillus_murinus
p_Actinobacteria
c_unidentified_Actinobacteria
o_Bifidobacteriales
g_Bifidobacterium
f_Bifidobacteriaceae
g_Prevotellaceae_UCG_001
g_Ileibacterium
s_Ileibacterium_valens
f_Ruminococcaceae
o_Oscillospirales
LDA SCORE (log 10)
q
Environmental_adaptation
Cell_motility
Metabolism_of_cofactors_and_vitamins
Signal_transduction
Cellular_processes_and_signaling
Nucleotide_metabolism
Replication_and_repair
Translation
Cancers
Genetic_information_processing
Transcription
Cellular_community_prokaryotes
Carbohydrate_metabolism
Ageing
Amino_acid_metabolism
Metabolism_of_terpenoids_and_polyketides
Metabolism_of_other_amino_acids
Lipid_metabolism
Infectious_diseases
Drug_resistance
Poorly_characterized
Xenobiotics_biodegradation_and_metabolism
Membrane_transport
Endocine_system
Cell_groth_and_death
Immune_system
Biosynthesis_of_other_secondary_metabolites
Energy_metabolism
Endocine_and_metabolic_diseases
Metabolism
Floding_sorting_and_degradation
Transport_and_catabolism
Nervous_system
Enzyme_families
Glycan_biosynthesis_and_metabolism

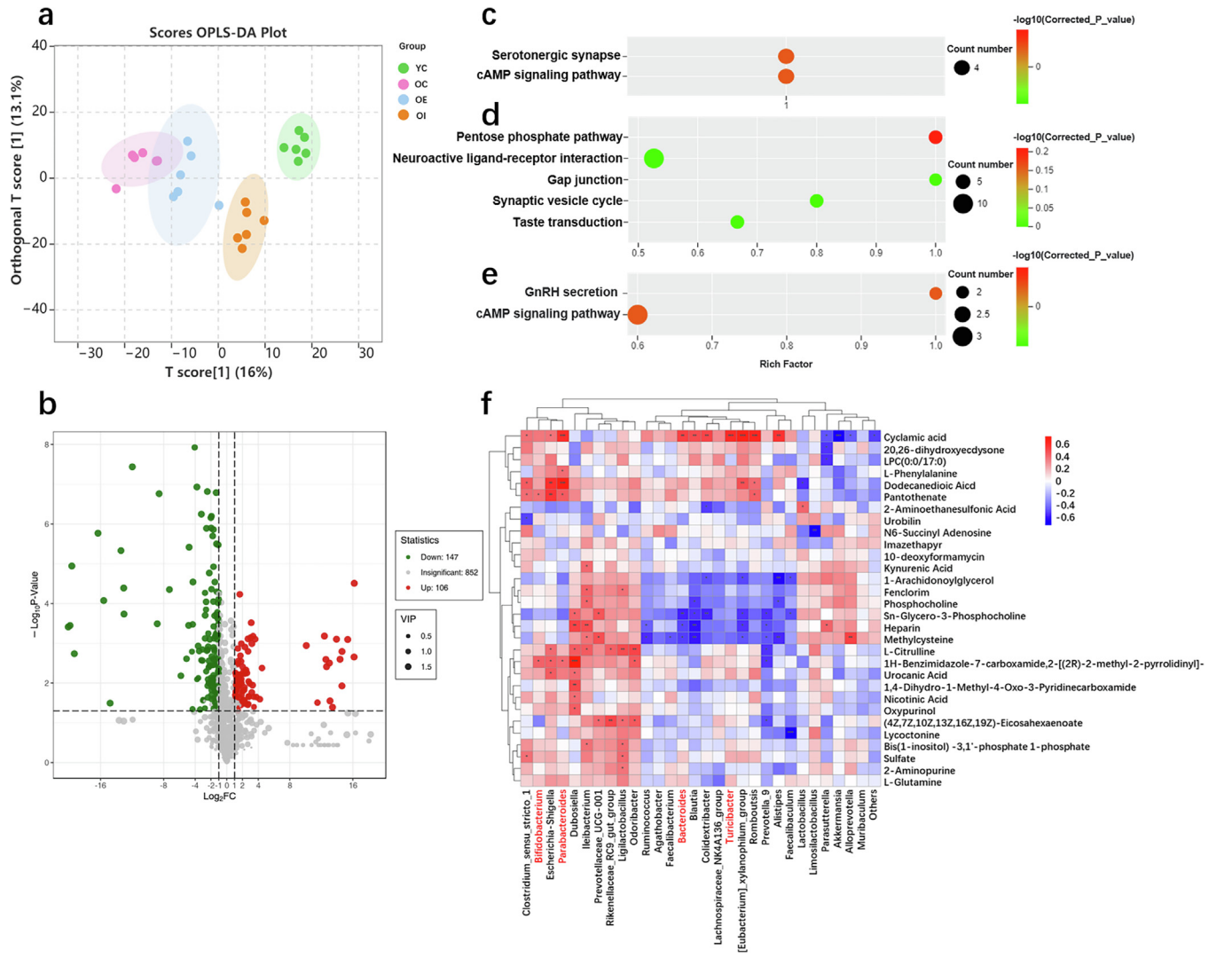



**Fig. 5.** The analysis of non-targeted metabolites in feces of mice from each group and its correlation with gut microbiota. (a) OPLS-DA analysis; (b) The volcano plot of differential metabolites between YC and OC groups; (c-e) The functional KEGG analysis of differential metabolites; (f) The correlation analysis between differential gut microbiota and metabolites. Columns are microorganisms, red indicates a positive correlation and blue indicates a negative correlation, as well as the greater absolute value of the correlation labeled with darker color.

aging mice. Changes in metabolites may induce or exacerbate the progression of diseases. Therefore, modulating gut microbiota may be essential to alter metabolites to intervene diseases.

*Exercise and irisin interventions regulated miRNAs and mRNAs in hippocampal tissues of aged mice*

The changes of miRNAs in hippocampal tissues of the mice from YC and OE groups were evaluated by miRNA sequencing to uncover the regulatory roles of miRNAs. Quality control, statistical analysis, and the analysis of the original off-machine data ($|\log_2 FC| \geq 1$, $p \leq 0.05$) found that compared with the YC group, 19 down-regulated and 12 up-regulated miRNAs in hippocampal tissues of the mice from the OC group were detected. Compared with the OC group, 7 down-regulated and 15 up-regulated miRNAs in the OE group, and 2 down-regulated and 8 up-regulated miRNAs in the OI group were detected, and a heatmap of the differential miRNAs in each group was established (Fig. 6a and Supplementary Fig. S3). The interaction network between partial miRNAs and genes in YC and OC groups was established to identify key miRNAs and corresponding target genes (Fig. 6b), and to list the significant differences in miRNAs and predicted target genes between each group (Supplementary Table S2-4). KEGG pathway enrichment of the predicted target genes of miRNAs in YC and OC groups was used to identify corresponding signal pathways, including ErbB,

**Fig. 4.** The 16S rRNA analysis and function prediction of gut microbiota in mice from each group. (a) The number of OTUs in mice from different groups; (b, c) The species compositions at the phylum and genus levels; (d-f) The TOP10 significantly different microorganisms at the phylum level in each group; (g-i) The TOP10 significantly different microorganisms at the genus level in each group; (j) PCA of fecal microorganisms in mice from each group; (k-n) Observed species, ACE, Shannon, and chao1; (o, p) The markers of LEfSe and differential gut microbiota; (d) Tax4Fun analysis and functional clustering of gut microbiota.

MAPK, cAMP, glutamatergic synapse, Toll-like receptor, Notch, Wnt, mTOR, Ras, apoptosis, insulin, mitophagy, NF-κB, TNF-α, and other signal pathways, with the involvement of inflammation and aging-induced neurodegenerative diseases (Fig. 6c), and to list the enrichment of KEGG pathways for miRNAs and predicted target genes between each group (Supplementary Table S5-7).

Similarly, after extracting total RNA of hippocampal tissues, mRNA sequencing was also carried out to screen differential mRNAs ($|\log_2 FC| \geq 1$, $p \leq 0.05$) between groups after statistical analysis of the effective data (transcripts per kilobase million, TPM). Compared with the YC group, 64 up-regulated and 28 down-regulated mRNAs in the OC group were detected. Compared with the OC group, 50 down-regulated and 11 up-regulated mRNAs in the OE group, and 79 down-regulated and 9 up-regulated mRNAs in the OI group were screened. The significantly differential genes were visualized (Fig. 6d, and Supplementary Table S8-10). Differential genes were enriched by KEGG (YC group vs. OC group), with major involvement in Toll-like receptor signal pathway, vascular smooth muscle contraction, dopaminergic synapse, NF-κB, cAMP, B cell receptor, Ras, longevity regulation, T cell receptor, TNF-α, insulin signaling, MAPK and other signal pathways (Fig. 6e), and the KEGG pathway enrichment of each group (Supplementary Table S11-13). Joint analysis was performed based on miRNA and mRNA sequencing, and differentially enriched pathways between different groups were predicted after KEGG enrichment analysis (Fig. 6f). Among them, the opposite signal pathways enriched in YC and OC groups could be observed that the differential pathways were mainly involved in ErbB, Toll, neurotrophin, circadian rhythm, aminoacyl-tRNA biosynthesis, apoptosis, Fc receptor of immunoglobulin E (Fc epsilon RI), prolactin, GnRH, glycerophospholipid metabolism, estrogen, NF-kappa B, retrograde endocannabinoid signaling, T cell receptor, TNF, natural killer cell-mediated cytotoxicity, insulin, phospholipase D, Hippo, cell adhesion molecules (CAMs), protein processing in the endoplasmic reticulum, axon guidance, focal adhesion signaling pathway, actin cytoskeleton, Ras, and MAPK signal pathways. The enrichment of KEGG pathways for the joint analysis of miRNAs and mRNAs between each group were also listed (Supplementary Table S14-16).

From above results, Toll, inflammation, and insulin signal pathways may be the major mechanisms involved in the changes of hippocampal tissues from aged mice upon exercise and irisin interventions, suggesting the "gut-brain" crosstalk efficiency.

## Correlation analysis between gut microbiota and miRNAs and mRNAs in hippocampal tissues of aged mice upon exercise and irisin interventions

To further explore the role of exercise and irisin interventions in the "gut-brain" axis, spearman correlation analysis for gut microbiota, miRNAs and mRNAs in hippocampal tissues between OC group and other groups were conducted to reveal a significant difference in *A2*, *Rikenella, Bifidobacterium*, *Parabacteroides*, *Monoglobus*, *Clostridium_sensu_stricto_1*, *Colidextribacter, Lachnolcostridium* and *Christensensensellaceae_R-7 group* at the genus level (Fig. 7a-b), and 46 differential miRNAs (Fig. 7c-d). Similarly, the correlation analysis between differential gut microbiota and 46 differential miRNAs was achieved (Fig. 7e). *A2*, *Rikenella*, *Bifidobacterium*, *Parabacteroides* and *Christensenellaceae_R-7_group* were significantly positively correlated with miR-1264-5p, miR-1264-3p, miR-1298-5p, miR-1912-3p, miR-204-5p, miR-211-5p, miR-34c/b-5p, miR-448-3p, miR-467d-5p, miR-467c-5p, novel-695, miR-196a-5p, and negatively correlated with novel-72a, novel-43u, miR-1957a, novel-420, miR-206-3p, novel-428a/b, novel-43, novel-869a/b/c, miR-130b-3p, miR-141-3p, miR-1251-5p and miR-200c-3p. *Lachnolcostridium* was positively correlated with miR-1264-5p, miR-1264-3p, miR-1298-5p, miR-1912-3p, miR-204-5p, miR-211-5p, miR-34c/b-5p, miR-448-3p, miR-1969, miR-1195, miR-196a/b-5p, novel-915, miR-467c/d-5p, novel-695, novel-95a/b/c/d, novel-67, miR-200b-5p, novel-101a/b, miR-10b-5p, miR-874a/b, novel-954, and significantly negatively correlated with novel-945, novel-730, novel-72a, novel-43u, miR-1957a, novel-420, miR-206-3p, novel-428a/b, novel-43, novel-869a/b/c, miR-130b-3p, miR-141-3p, miR-1251-5p, and miR-200c-3p. *Monoglobus* was positively correlated with miR-467d-5p, miR-467c-5p, novel-695 and miR-196a-5p, and negatively correlated with novel-72a, novel-43u and miR-1957a, *Clostridium_sensu_stricto_1* was negatively correlated with miR-34b-5p, novel-730 and miR-196a-5p, and positively correlated with novel-95b/c, novel-95a/c/d, miR-1957a, miR-1251-5p and miR-200c-3p. *Colidextribacter* was negatively correlated with miR-1264-5p, miR-1264-3p, miR-1298-5p, miR-1912-3p, miR-204-5p, miR-211-5p, miR-34c-5p, miR-448-3p, miR-34b-5p and novel-945, and positively correlated with novel-67, novel-101a, miR-141-3p, miR-1251-5p and miR-200c-3p. *Clostridium_sensu_stricto_1* was negatively correlated with miR-34b-3p, novel-730, miR-196a-5p, and positively correlated with novel-95a/b/c/d, miR-1957a, miR-141-3p, miR-1251-5p and miR-200c-3p.

At the same time, the differentially expressed gene transcripts in the OC group were screened and 747 genes were differentially expressed in the YC group, 681 genes in the OE group, and 1068 genes in the OI group; among them, there were 35 common genes (Fig. 7f-g), with correlation with gut microbiota in YC, OE, and OI groups, which revealed the significant difference from the OC group (Fig. 7h). It was found that *Parabacteroides* was positively correlated with *Cpne1*, *Pak3*, *Sema4a*, *Wdr48*, *Adra1a*, *Hspa8*, *Ly6e*, *Mtm1*, *Dpagt1*, *Hp1bp3*, *Clvs1* and *Abcb8*, and negatively correlated with *Gbp6*, *U2af2*, *A930004D18Rik*, *Cxcr4*, *Ica1*, *Mecp2*, *Caln1*, *Hcfc2*, and *Scmh1*; *Bifidobacterium* was positively correlated with *Wdr48*, *Adra1a*, *Hspa8*, *Ly6e* and *Mtm1*, and negatively correlated with *Hcfc2* and *Prdm16*; *A2* was negatively correlated with *Clpb*, and positively correlated with *Tbc1d24*; *Rikenella* was negatively correlated with *Magi2* and *Akt1s1*, and positively correlated with *Mndal*; *Monoglobus*, *Christensensensellaceae_R-7*, *Clostridium_sensu_stricto_1*, *Colidextribacter*, and *Lachnolcostridium* were positively correlated with *Cpne1*, *Pak3* and *Sema4a*, and negatively correlated with *Scmh1*. These results indicated that the correlation analysis of gut microbiota and miRNAs and mRNAs in hippocampal tissues of the mice from YC, OE, and OI groups was different from OC group, which may be the possible mechanisms for regulating gene expression in hippocampal tissues from the perspective of gut flora.

### *Exercise and irisin interventions alleviated inflammatory responses and TLR4/MyD88 signal pathway-mediated insulin resistance*

From the analysis of fecal microbes, non-targeted metabolites, and screened miRNAs and mRNAs in hippocampal tissues, the screened signal pathways were predicted and enriched to be correlated with aging, inflammation, and neurodegenerative diseases. Toll receptor and insulin signal pathway were enriched with miRNAs and mRNAs through the analysis of KEGG pathways in hippocampal tissue. Lipopolysaccharide-binding protein (LBP) activating factor and IL-1β, IL-6 and TNF-α from each group were detected. These results showed that LPS, LBP, IL-1β, IL-6, and TNF-α in serum of the mice from the OC group were significantly increased ($p = 0.0405$, $p = 0.0287$, $p = 0.0089$, $p = 0.0188$, $p = 0.0149$) when compared with the YC group, while LPS, LBP, IL-1β and TNF-α in serum of the mice from OE and OI groups were significantly decreased ($p = 0.0074$, $p = 0.0317$, $p = 0.0192$,

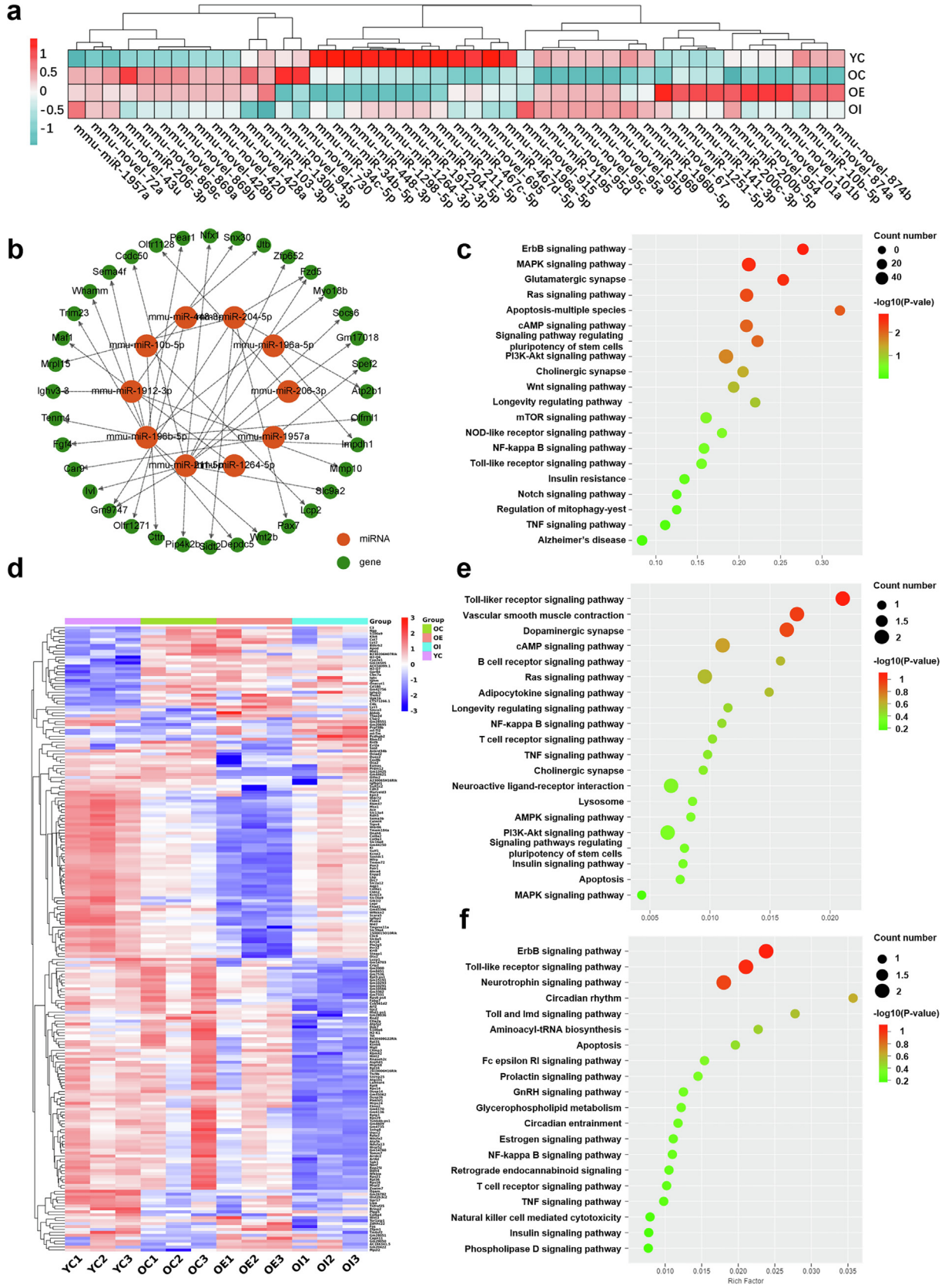



**Fig. 6.** Hippocampal miRNAs, mRNAs and joint analysis of the mice from each group. (a) Behavioral differential miRNAs, listed as groups; (b) Red indicates miRNAs and blue indicates target genes; (c) KEGG functional enrichment of differential miRNA target genes; (d) Heat map of differential genes in hippocampal tissues of the mice from each group. (e) KEGG functional enrichment of differential mRNAs; (f) The joint analysis of the difference between miRNAs and mRNAs. Red indicates a positive correlation, blue indicates a negative correlation, and darker color reveals higher correlation.

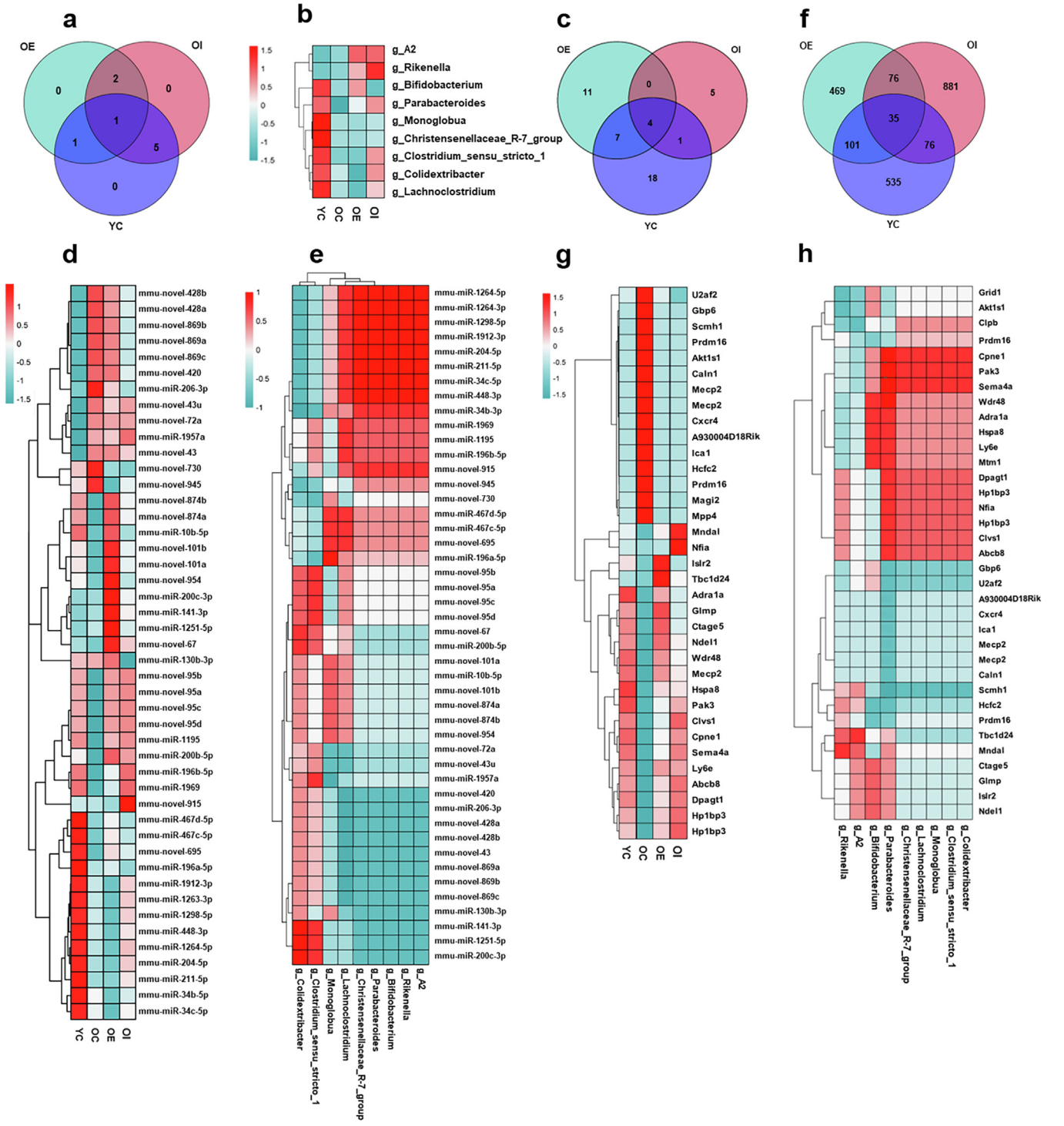



**Fig. 7.** The correlation analysis of gut microbiota and miRNAs and mRNAs in YC, OE, and OI groups differed from OC group. (a) The number of gut microbiota in YC, OC, and OE groups differed from OC group; (b) The differential microbiota in each group at the genus level; (c, d) The number and names of miRNAs in YC, OC, and OE groups differed from OC group; (e) The clustering heatmap of correlation between miRNAs and genus-level gut microbiota in YC, OC, and OE groups differed from OC group; (f) The number and names of differential genes in YC, OC, and OE groups differed from OC group; (h) The clustering heatmap of the correlation between genus-level differential gut microbiota and genes. Red indicates a positive correlation, blue indicates a negative correlation, and darker indicates a more significant correlation.

$p$ = 0.0219; $p$ = 0.0001, $p$ = 0.0055, $p$ = 0.0024, $p$ = 0.0430, $p$ = 0.0384) (Fig. 8a). The upstream and downstream of TLR4-mediated inflammatory signal pathways were verified by qRT-PCR. *TLR4*, *MyD88*, *NF-κB*, *IL-1β*, *IL-6*, and *TNF-α* were significantly up-regulated in hippocampal tissues of the mice from the OC group ($p$ = 0.0039, $p$ = 0.0084, $p$ = 0.0254, $p$ = 0.0010, $p$ = 0.007, $p$ = 0.0125) when compared with the YC group; compared with the OC group, *TLR4*, *MyD88*, *NF-κB*, and *IL-1β/6* were significantly decreased in the OE group ($p$ = 0.0019, $p$ = 0.0424, $p$ = 0.0153, $p$ = 0.0056, $p$ = 0.0006), and *LBP*, *TLR4*, *MyD88*, *JNK*, and *IL-1β/6* were significantly reduced in the OI group ($p$ = 0.0425, $p$ = 0.0016, $p$ = 0.0123, $p$ = 0.0079, $p$ = 0.0036, $p$ = 0.001) (Fig. 8b). Moreover, during detecting the inflammatory state of hippocampal tissue in each group were stained by Iba1, GFAP and p-NF-κB p65, com-

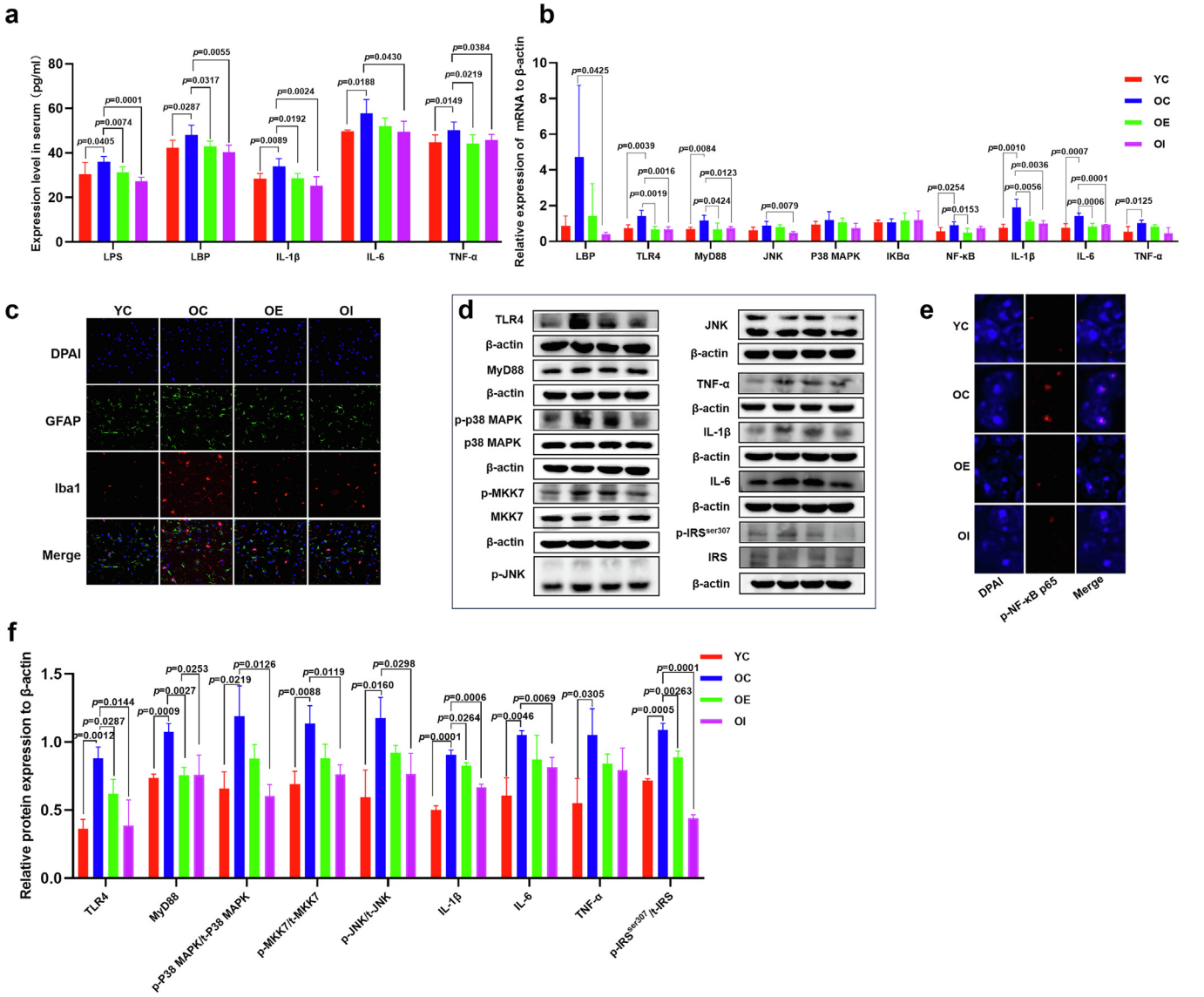



**Fig. 8.** The serum inflammatory factors, hippocampal TLR4/MyD88 signal pathway-related genes, and corresponding proteins in hippocampal tissues of the mice froom each group. (a) Serum levels of inflammation-related factors in different groups; (b) Hippocampal TLR4/MyD88 signal pathway-related inflammatory mRNA levels in each group; (c) Immunofluorescent staining of Iba1 and GFAP in hippocampal tissues of the mice from each group; (d) Corresponding inflammation levels in hippocampal tissues of the mice from each group evalauted by protein expression of p-NF-κB p65 through immunofluorescence staining; (e) Statistical analysis for expression levels of corresponding inflammatory proteins in hippocampal tissues of the mice from each group.

pared with YC group, the activation levels of hippocampal microglial cells and NF-κB p65 were increased in the OC group, but the activation of hippocampal microglial cells and NF-κB p65 in OE and OI groups were decreased when compared with the OC group (Fig. 8c and e).

Furthermore, the expression of proteins associated with the inflammatory activation status was evaluated by Western blot. TLR4, MyD88, p-p38 MAPK/p38 MAPK, p-MKK7/MKK7, p-JNK/JNK, IL-1β/6, TNF-α, p-IRS$^{ser307}$/IRS were significantly increased ($p$ = 0.0012, $p$ = 0.0009, $p$ = 0.0219, $p$ = 0.0088, $p$ = 0.0160, $p$ = 0.001, $p$ = 0.0046, $p$ = 0.0305, $p$ = 0.0005) in the OC group when compared with the YC group (Fig. 8d and f). Compared with the OC group, TLR4, MyD88, IL-1β and p-IRS$^{ser307}$/IRS were decreased ($p$ = 0.0287, $p$ = 0.0027, $p$ = 0.0264, $p$ = 0.0263) in the OE group, and TLR4, MyD88, p-p38 MAPK/p38 MAPK, p-MKK7/MKK7, p-JNK/JNK, IL-1β/6 and p-IRS$^{ser307}$/IRS were significantly decreased ($p$ = 0.0144, $p$ = 0.0253, $p$ = 0.0126, $p$ = 0.0119, $p$ = 0.0298, $p$ = 0.0006, $p$ = 0.0069, $p$ = 0.001) in the OI group. The above results indicated that hippocampal tissues of aged mice might induce neuronal inflammation and insulin resistance mediated by TLR4/MyD88 signal pathway through the activation of microglia by LPS. Exercise and irisin interventions could inhibit LPS in the body, alleviate the activation of microglial cells, and suppress hippocampal inflammation and insulin resistance.

## Discussion

Exercise-modulating gut microbiota may be related to increasing irisin levels in the body. The sequencing of fecal 16S rRNA, nontargeted metabolites, and hippocampal miRNAs and mRNAs was conducted to further validate the effects of exercise and irisin-regulated gut microbiota and metabolites on miRNAs and genes in hippocampal tissues. The gut microbiota, genes, and signal pathways involved in these analyses may become potential intervention targets for improving cognitive capacity, among which, the activation of the TLR4 signal pathway in brain tissue mediated

by increased LPS due to the imbalanced gut microbiota may be an important regulator. Exercise suppresses neuroinflammation mediated by TLR4/MyD88 signal pathway in brain tissues to enhance cognitive capacity, which may be related to exercise-induced irisin in the body.

Aging can lead to impaired cognitive capacity, while drugs, exercise, and nutritional supplements are currently central interventions. To better understand the effect of exercise on aging-induced cognitive impairment, moderate-intensity aerobic and anaerobic training as possible within a week with a duration of 45–60 min for each training period reveals a significant improvement on cognitive performance in older adults over the age of 50 [24], and aerobic exercise is superior in cognitive improvement [25]. Therefore, 45-min aerobic treadmill running with 5 times a week and a training period of 3 consecutive months was provided for 21-month-old mice to further explore the molecular mechanisms of aerobic exercise-induced cognitive improvement during aging process in the present study. The improvement of learning and memory capacity upon exercise interventions may be correlated with increased function and number of neurons. Meanwhile, the observation with hippocampal HE, Nissl and NeuN staining, and TEM examination revealed the increased neuronal number, synaptic arc length, and PSD thickness of neuronal synapses after exercise intervention, and the synaptic gap was decreased significantly [26], including the critical role of gut microbiota [27].

There is a significant difference in irisin level across different models. Regression analysis of disease states shows that plasma irisin level is positively correlated with normal and mildly impaired cognitive capacity of patients without AD. In contrast, higher irisin level in AD patients may have smaller hippocampal volume [28], indicating that the occurrence of the disease may be a feedback response from the increased irisin to slow down the disease progression and the depletion of irisin secretion when the disease state is irreversible. Relevant studies have also confirmed that exercise can enhance cognitive capacity due to the optimized gut microbiota [29,30]. In the present study, serum irisin levels in mice subjected to exercise and irisin interventions are significantly higher than the aging model mice, which is consistent with previous results [31]. Moreover, the direct injection of recombinant irisin has the stronger effect than exercise intervention. However, the studies on irisin-mediated optimization of gut microbiota are limited, and the regulation of gut microbiota by irisin is only reported in colitis and ischemia–reperfusion injury models [11,13]. It is worth noting that although exercise does not show a significantly regulatory effect on gut microbiota, it still shows a significantly reversal cognitive capacity during aging process, suggesting exercise-induced multiple benefits. Similarly, exercise can effectively regulate gut microbiota to prevent gut barrier damage [32]. However, the studies in mouse aging models are still limited. The limited regulation capacity to gut microbiota may be associated with lower exercise capacity and excessive oxidative stress in older mice [33,34]. In contrast, excessive exercise intensity may impact the structure and function of gut microbiota in the elderly population [35]. Meanwhile, some studies have also shown that exercise can reshape gut microbiota although the reshaping capacity is limited [36]. In the present study, exercise has a regulatory effect on gut microbiota, and is weaker than irisin intervention, which may be due to irisin circulation during exercise intervention when compared with direct irisin intervention. These results suggest that moderate-intensity aerobic exercise has a limited role in regulating gut microbiota in aged mice or exercise-induced irisin does not reach up to the level for triggering gut microbiota.

Our 16S RNA sequencing data of gut microbiota have confirmed that aging can lead to a significant decrease in *F*/*B* ratio and abundance of *Actinobacteria*, *unidentified_Bacteria*, *Parabacteroides*, *Bacteroides*, *Turicibacter*, and *Bifidobacterium*, whereas exercise and irisin injection can reverse the decline in the abundance of gut microbiota that is closely related to cognitive capacity. Statistical analysis of gut microbiota at the phylum and genus levels has documented that *F*/*B* ratio shows a significant decrease in aged mice, with consistent results with previous studies [37,38], and the *F*/*B* ratio decline may be also due to aging stages, and races [39–41]. In a previous study on gut microbiota and cognition in neurologically healthy elderly, it has found that *Firmicutes* is positively correlated with memory capacity, but *Bacteroidetes* is negatively correlated [42]. The fecal microbiota test of Amyotrophic lateral sclerosis patients with cognitive impairment and Amyotrophic lateral sclerosis patients with normal cognition also shows a significant increase in *F*/*B* ratio [43], which is consistent with the decrease in *F*/*B* ratio observed during aging process. In rats with memory impairment, the traditional Chinese herbal prescription Dangshen Yuanzhi Powder also can reverse the decrease in *F*/*B* ratio while improving memory capacity [44]. In the present study, exercise and irisin interventions reversed this ratio and showed the improved cognitive capacity, further confirming that *F*/*B* ratio may be a potential factor of cognitive capacity. However, in obesity models with cognitive impairment caused by diabetes, *F*/*B* ratio shows a significant increase [45,46], which may be related to unnatural aging and exogenous food or drug intervention. A significant increase in *Actinobacteria* has been found in both AD and elderly cognitive impairment populations [47–49]. However, *Actinobacteria* reveals an significant increase in studies using Triphala to improve cognition in AD mice [50], and *Actinobacteria* was a significant decrease in abundance with increasing age [51], which is also consistent with our results. In addition, the studies on the relationship between intestinal microbial changes and cognition in AD people have also confirmed that the abundance of *Actinobacteria* is significantly reduced [52]. Significant reduction in *Actinobacteria* has been found in 5XFAD mice with severe cognitive decline when compared with wild-type mice at the same age [53]. In another study, *Actinobacteria* is directly linked to cognitive capacity [54]. The traditional Chinese herb Jiedu Yizhi prescription can significantly improve cognitive capacity of APP/PS1 mice, and can also suppress the TLR4/NF-κB signal pathway in the brain through inhibiting *Actinobacteria*, further suggesting that *Actinobacteria* may be involved in regulating signal pathways for controlling cognitive capacity [55]. In chronic neuropathic pain models, a selective decrease in gut flora accompanied by an increase in *Actinobacteria* may contribute to the occurrence of cognitive impairment [56]. PD also shows a rapid decline in cognitive level during the development process of the disease. A 3-year follow-up study has found that the reduction of *Actinobacteria* is closely related to accelerated cognitive deterioration [57]. *Actinobacteria* abundance also shows a significant increase when administrated with the cognitively beneficial Enoki mushroom polysaccharide [58]. However, *Actinobacteria* in the studies of aging and cognition fields has received attention only in recent years [59]. Although there is some controversy, more studies on its beneficial cognitive improvement may be needed to further confirm.

Based on our study, *Parabacteroides* reveals a significant decrease during aging process, while exercise and irisin interventions can reverse its aging-induced reduction. Similarly, a significant decrease in the abundance of *Parabacteroides* has also found in the elderly with mild cognitive impairment [60]. In hemodialysis patients with cognitive impairment, there is also a positive correlation between *Parabacteroides* and cognitive capacity [61]. Cognitive capacity can be enhanced by optimizing the compositions of gut flora upon probiotics supplementation, in which the abundance of *Parabacteroides* also shows a significant increase [62]. However, *Parabacteroides* show an increase in models of cognitive impairment after schizophrenia, stroke, and radiation-

induced cancers [63–65]. These opposite results may be related to different disease models, and the specific factors may require more in-depth study. The reduction of *Bacteroides* during aging process may be negatively correlated with body inflammation [66], and supplementing *Bacteroides* can inhibit inflammation levels [67]. Therefore, *Bacteroides* should have close association with cognitive improvement in the elderly [68], is positively correlated with cognitive capacity [69], as well as involved in neurodevelopment to improve infant cognitive capacity [70]. In clinical studies, fecal transplants can suppress cognitive decline due to the significant increase in *Bacteroides* [71]. Mendelian analysis of over 200,000 cognitive data and nearly 20,000 gut microbes has demonstrated that *Bacteroides* plays an active role in cognitive protection [72]. Some studies have shown a significant increase in *Bacteroides* by transplanting gut flora of AD persons into germ-free mice [73]. The detection of microbiota in people with dementia has also confirmed an increase in the abundance of *Bacteroides* as an independent factor in dementia [74].

In addition, the beneficial *Turicibacter* is also significantly reduced in aging mice, while exercise and irisin interventions rescue this deleterious reduction. Supplementing *Turicibacter* could delay aging process to a certain extent [75], and a significant decrease in *Turicibacter* abundance is also observed in APP/PS1 mice with poor cognitive capacity [76]. Dietary methionine restriction as a diet intervention for anti-aging and suppressing aging-induced cognitive impairment can stimulate cognitive capacity through significantly increasing the abundance of *Turicibacter* [77]. *Bifidobacterium* exhibits anti-aging effects on promoting cognitive capacity and metabolic level of AD mice [78,79], which is possibly correlated with neuron regeneration [28]. In a randomized controlled trial on the elderly, it is found that exogenous supplementation of *Bifidobacterium* can significantly improve the learning and memory capacity of elderly population [80]. Pregnant mice supplemented with omega-3 exhibit a significant increase of *Bifidobacterium* and enhanced cognitive capacity in their offsprings [81]. Aged mice and humans supplemented with *Bifidobacterium* have also found that cognitive function is significantly enhanced, which may be related to the improvement in the gut environment and barrier and inflammation levels [82,83]. Moreover, middle-aged mice supplemented with *Bifidobacterium* show improved cognitive capacity and metabolic function, further confirming its impact on brain function [84,85]. Supplementing *Bifidobacterium* has shown to modulate gut flora when rescuing mild cognitive impairment, with the involvement in improving synaptic plasticity through up-regulating brain-derived neurotrophic factor (BDNF), fibronectin type III domain-containing protein 5 (FNDC5), and postsynaptic density protein 95 (PSD-95) in the brain [86]. After exercise and irisin interventions in the present study, the level of *Bifidobacterium* in aged mice reveals a significant increase accompanied by the improvement of brain structure and cognitive capacity, further suggesting that exercise or exercise-induced irisin may promote the optimal balance in the abundance of beneficial bacteria.

The change in gut microbiota can induce corresponding change of the metabolites in feces. The fecal metabolites have significant differences in the OPLS-DA scores of the metabolites from different interventions. *Bacteroides, Turicibacter,* and *Bifidobacterium* at the genus level are significantly reduced in feces of aged mice, which can be reversed by exercise and irisin interventions. *Bifidobacterium* is significantly positively correlated with 1H-2-(2R)-2-methyl-2-pyrrolidinyl-benzimidazole-7-carboxamide, and pantothenate, among which 1H-2-(2R)-2-methyl-2-pyrrolidinyl-benzimidazole-7-carboxamide has limited research, while pantothenic acid is involved in improved cognitive capacity. Pantothenic acid is also known as vitamin B5, and the deficient intake of pantothenic acid may lead to cognitive decline [87]. In the analysis of human cerebrospinal fluid components, it is found that pantothenic acid is significantly positively correlated with aging [88], and abnormal change in pantothenic acid is also found in gut flora of normal people and people transitioning from MCI to dementia [89]. *Turicibacter* is significantly positively correlated with cyclamic acid. However, the studies on the role of cyclomic acid in cognition seem to be lacking. *Bacteroides* is significantly negatively correlated with *sn-glycero*-3-phosphocholine, methylcysteine and heparin, and positively correlated with cyclamic acid. Sn-*glycero*-3-phosphocholine (synonym choline alfoscerate) as an acetylcholine precursor in improving cognition has been confirmed by many studies. Sn-*glycero*-3-phosphocholine has a positive effect on cognitive improvement in natural aging and different stages of AD populations [90,91], and is even considered a cognitive enhancer. A *meta*-analysis involving 449 population-based trials has demonstrated that phosphocholine supplementation can significantly improve cognition [92]. However, the relationship between methylcysteine, heparin, and cyclohexanoic acid and cognition has been less studied.

To explore the relationship between gut microbiota and miRNAs and mRNAs in hippocampal tissues, differential gut microbiota between YC, OE, OI, and OC groups at the genus level are screened. Among differential miRNAs, miR-206-3p is significantly up-regulated in the OC group and down-regulated in YC, OC and OI groups at a certain degree. The miR-206-3p is directly involved in regulating BDNF [93], which may become a potential marker for diagnostic and treatment tools for AD [94]. In addition, the relative expression of miR-103-3p and miR-103b-3p was higher in OC and OE groups. Relevant studies have shown that lower levels of miR-103-3p in human serum are independently associated with cognitive impairment [95]. Based on sequencing data of AD patients and normal populations, miR-103-3p can interact with most of the key genes screened to predict AD [96]. MiR-103-3p in the OE group seems to be consistent with that in the OC group, but relevant studies have confirmed that exercise may cause the increase of miR-103-3p to a certain extent [97], but the studies between miR-103b-3p and cognitive capacity have not been reported. In transcriptome sequencing, we have found that some genes closely correlated with cognition are significantly different between YC and OC groups, such as *Cst7*, *Klk6*, *Slc13a4*, *Trem2*, *Slc16a9*, *Cldn1*, and *Apod*. The *Cst7* gene is up-regulated in the OC group, but down-regulated in the YC and OI groups, and is also associated with cognitive decline [98], while the inhibition of *Cst7* shows cognitive deficits [99]. As a gene closely related to AD, the expression of *Klk6* can lead to neuronal degeneration [100], and *Klk6* has been found to be significantly elevated in plasma of AD persons, and may become a biomarker for predicting the severity of dementia AD [101]. *Slc13a4* has sodium-sulfate symporter activity, and the deletion of the *Slc13a4* gene causes a significant decrease in the learning and memory capacity of mice, but in contrast, *Slc13a4*$^{+/-}$ mice show an increase in the appearance of hippocampal neurons [102]. *Trem2* has been found to be related to AD in many studies and is highly expressed in AD. Inhibiting *Trem2* has also become an important target to alleviate cognitive decline [103,104]. *Slc16a9* methylation mediates the effects of prenatal alcohol exposure on neonatal cognitive and attention-related deficits [105]. In the blood–brain barrier endothelial cells of aged mice, TLR4 activation is accompanied by a decrease in *Cldn1*, while the downregulation of TLR4 shows an increase in *Cldn1*, and *Cldn1* is positively correlated with cognition [106]. Leukoaraiosis patients with cognitive impairment also show a significant decrease in *Cldn1* [107]. Moreover, the inhibition of *ACE* and *ACE* heterozygous mice show an exacerbation of AD symptoms [108], and the underlying mechanism may be related to the degradation of β-amyloid protein [109]. However, there are many studies showing that *ACE* is not correlated with cognition [110], or that blocking *ACE* can

alleviate further deterioration of cognition [111]. Therefore, the relationship between *ACE* and cognition may need further exploration. *Apod* shows the up-regulation with the extension of age [112], also shows high expression in AD patients [113] and a negative correlation with cognitive capacity [114]. *Gm28294* and *C5ar2* are significantly down-regulated in the OC group and increased in YC, OE and OI groups. Unfortunately, there are no reports on *Gm28294* in the literature. *C5ar2* may play a neuroprotective role in AD and delay the occurrence of AD, complement and astrocyte-related genes to a certain extent [115]. *Gpr17* and *Fos* are significantly down-regulated in the OC group and up-regulated in the OE group. However, some studies have shown that knocking out and inhibiting *Gpr17* exhibits positive effects on cognition [116,117], which is contrary to our results. *Fos* gene plays an important role in spatial memory [118]. The c-Fos as one of the *Fos* gene members is dysregulated in AD patients [119], and is also significantly down-regulated in aging mice with cognitive deficits [120], while running training can significantly up-regulate its expression in hippocampal tissues [121], which is consistent with our results.

*U2af2*, *Gbp6*, *Scmh1*, *Prdm16*, *Akt1s1*, *Caln1*, *Mecp2*, *Cxcr4*, *A930004D18Rik*, *Ica1*, *Hcfc2*, *Prdm16* and *Magi2* genes are up-regulated in the OC group, while *Adra1a*, *Glmp*, *Ctage5*, *Ndel1*, *Wdr48*, *Tenm4*, *Hspa8*, *Pak3*, *Clvs1*, *Cpne1*, *Sema4a*, *Ly6e*, *Abcb8*, *Dpagt1* and *Hp1bp3* are down-regulated in the OC group, which may be the important predictors of aging. Based on the correlation analysis of gut microbiota *A2*, *Rikenella*, *Bifidobacterium*, *Parabacteroides*, *Monoglobus*, *Christensensenselaceae_R-7*, *Clostridium_sensu_stricto_1*, *Colidextribacter* and *Lachnolcostridium* at the genus level and miRNAs and mRNAs, gut microbiota has a significant potential role in influencing hippocampal miRNAs and mRNAs to participate in aging-induced neurological diseases (Fig. 6h).

Inflammation-induced insulin resistance is one of the major inducers of aging-induced cognitive impairment, which may be closely correlated with the increased LPS and LBP in the body. Both exercise and irisin interventions have been confirmed to exert anti-inflammatory effects with the focus on TLR4/MyD88 [14,122,123]. Under normal physiological conditions, LPS in gut microbiota can maintain homeostasis at a certain level. At aging and disease states, abnormal changes in gut microbiota may lead to the release of a large amount of LPS, thereby aggravating the inflammatory response of the whole body and organs, and even inducing insulin resistance and the occurrence of diseases. TLR4 signal pathway is the primary inflammatory signal for LPS-induced LBP activation. In the present study, suppressing TLR4 signal pathway in hippocampal tissues of aged mice upon exercise and irisin interventions can suppress inflammation. Similarly, the significant up-regulation of *TLR4*, *MYD88*, *IL-1β*, and *IL-6* genes in hippocampal tissues of the elderly has also been confirmed by human experiments [124,125]. TLR4 may mediate the transduction of IL-1β signal pathway in hippocampal tissues of aged mice. The absence of TLR4 can offer aged mice for better learning and memory functions [20,126], which may be closely correlated with the suppression of inflammation and the mitigation of neuronal disorders [127], illustrating that the activation of TLR4 may be one of the important causes of insulin resistance and accelerating cognitive impairment during aging process [128]. During natural aging or drug-induced aging process, p-p38 MAPK in hippocampal tissues can be triggered to a certain extent [129,130], and aerobic exercise can significantly result in its down-regulation [131], which is consistent with our study, suggesting an obviously positive anti-inflammation efficiency and enhanced insulin sensitivity upon exercise and irisin interventions. However, the effect of irisin intervention on inhibiting hippocampal neuroinflammation and insulin resistance is better than that of exercise intervention, which may be related to the fact that the irisin level in aging mice induced by moderate exercise is not enough to optimize gut microbiota for suppressing inflammation. The specific mechanism needs to be further clarified.

In the present study, comprehensive testing of gut flora, non-targeted metabolites, hippocampal miRNAs and mRNAs, blood-related indicators, and hippocampal tissue samples shows that irisin intervention is more effective than exercise. Exercise-induced regulation of the gut-brain axis may be closely related to resultant endocrine, metabolite and physiological changes involved in regulating gut flora. Therefore, exercise-induced irisin can modulate gut microbiota to improve cognition, which is also a new way to uncover exercise to improve cognition. Analyzing how exercise regulates gut flora and participates in disease improvement may be a focus of future research. At present, it has been confirmed that irisin is positively correlated with cognitive capacity of AD persons with cognitive impairment [132], and exercise plays a role in enhancing cognition by increasing irisin levels [13]. However, exercise seems to be a reference only for people with normal physical activities, but not for people with cognitive impairment, physical disabilities or those who are unable to accomplish exercise. Therefore, direct intervention with irisin to achieve the mimic effects of exercise on health promotion and disease prevention or rehabilitation is the ultimate goal that we are currently pursuing. Unfortunately, there are currently no experimental reports on irisin interventions in humans, but exercise [13], nattokinase [133], low-intensity pulse [134], solanum melongena extract [135] can trigger the circulating level of irisin in the body. Therefore, increasing the circulating level of irisin in the body through exogenous interventions targeting the body's irisin may also be a potential strategy for disease interventions in the future. It is worth noting that irisin appears to vary in different diseases, and if used in human treatment, more detailed screening of body functions and diseases may be required. The collection of data from larger samples of people with different ages and diseases and the exploration of human-related experiments may be a breakthrough in promoting the clinical value of irisin.

## Conclusion

Exercise-reversed cognitive impairment of aging mice may be correlated with the generation and secretion of exercise-induced irisin and the irisin-mediated optimization of gut microbiota and metabolites through suppressing TLR4/MyD88 signal pathway and insulin resistance. This finding may provide a new reference for exercise-mediated gut microbiota to enhance cognitive capacity during aging process. Unfortunately, the beneficial effect and underlying mechanisms of irisin on optimizing the balance of gut microbiota are not fully validated. Moreover, irisin gene knockout to affect the regulation of gut microbiota and cognitive capacity may need to be further verified.

## Compliance with Ethics Requirements

All experiments involving animals were conducted according to the ethical policies and procedures approved by Institutional Animal Care and Use Committee of Wuhan Sports University with the number of S087-21-05D, and all experiments involving animals were complied with the internationally recognized 3R principles.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2024.10.031.

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