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Food Science and Human Wellness

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

Integration of gut microbiota and metabolomics analysis to unravel the neuroprotective effects of hydroxy- α -sanshool on Alzheimer's disease

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ARTICLE INFO

Article history:

Received 24 November 2023

Received in revised form 13 December 2023

Accepted 28 March 2024

Keywords:

Hydroxy- α -sanshool

Alzheimer's disease

Oxidative stress

Apoptosis

Gut microbiota

Metabolomics analysis

ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disease, and oxidative stress induced by amyloid β -protein ($A\beta$) deposition is the key cause of neuronal apoptosis in AD patient. Hydroxy- α -sanshool (HAS) is the predominant flavour substances in the pericarp of *Zanthoxylum bungeanum* Maxim. (Rutaceae family), which is a known condiment food and herbal medicine in China. We attempt to comprehensively elucidate the pathway and related mechanisms of HAS therapy on AD through *in vivo* and *in vitro* experiments. The results showed that HAS could inhibit cell apoptosis, suppress the reactive oxygen species (ROS), and increase the antioxidative enzymes in $A\beta_{1-42}$ induced HT22 cells. *In vivo*, HAS attenuated the learning and memory impairment in senescence-accelerated mouse prone 8 (SAMP8) mice, and protected neuronal cells in hippocampus. Interestingly, the 16S rRNA gene sequencing results showed HAS could restore the richness and diversity of the intestinal microbiota, reduce the abundance of *Lactobacillus*, and increase the abundance of *Bacteroides* and *Parabacteroides*. Moreover, the contents of 10 metabolites were significantly changed after HAS treatment, which was beneficial to treat AD. In conclusion, HAS could inhibit apoptosis in nerve cells by attenuating $A\beta$ induced oxidative stress level, while it can also influence the metabolic pathways and affect the gut microbiota composition of AD mice.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by disorders in learning and cognition, accompanied by deterioration of the ability to perform daily activities^[1]. Importantly, AD patients account for approximately 60%

of all the dementia patients, while the cost AD treatment accounts for 0.65% of global GDP^[2]. Up to now, the available drugs used to treat AD mainly include acetylcholinesterase inhibitors (representing donepezil, rivastigmine and galantamine) and *N*-methyl-*D*-aspartic acid receptor antagonists (representing memantine)^[3]. However, the above drugs can only relieve partial symptoms temporarily, and cannot prevent the occurrence and development of AD. In addition, drugs mentioned above may abnormally increase neurotransmissions, which makes AD treatment more difficult^[3]. The hypotheses about AD pathogenesis mainly include the neuronal tangles caused by Tau protein hyperphosphorylation and the aggregation of amyloid

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Peer review under responsibility of Beijing Academy of Food Sciences.

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β -protein ($A\beta$). It is known that $A\beta$ deposition would lead to neuron loss, which is also the major pathological change in AD^[4]. In addition, it has been shown that apoptosis will be significantly increased in hippocampal neurons of AD patients, and is closely related to the deformation of neurons^[5]. Unfortunately, the researchers designed a series of small-molecule compounds, however most of them failed to achieve expected results.

In recent years, accumulating evidence lends support to the view that intestinal microbiota plays an important role in the pathogenesis and development of AD owing to the continuously improved sequencing technologies. Abnormal microbial composition can be detected in the gut of AD patients. Besides, it has been proven that intestinal microbiome disorders occur prior to $A\beta$ deposition and Tau protein accumulation^[6], which disrupts the metabolism of fats, amino acids and carbohydrates in the body^[7-8]. Subsequently, these microbial metabolites, in particular short-chain fatty acids, can lead to $A\beta$ deposition, thus accelerating the development of AD^[9]. The imbalance of intestinal microbiome can induce the secretion of lipopolysaccharide and amyloid protein, thereby increasing intestinal and blood-brain barrier permeability, and ultimately impairing neurodevelopment^[10-11]. In addition, Firmicutes, *Proteus* and their metabolites can also participate in signal pathway transduction by regulating the expressions of the brain-derived neurotrophic factor (BDNF), thus affecting the occurrence of neuroinflammation^[12]. Therefore, remodelling the gut microbiome may be a feasible effective way for management of AD.

Hydroxy- α -sanshool (HAS) is the predominant flavour substance in the pericarp of *Zanthoxylum bungeanum* Maxim. (ZBM) (Rutaceae family), which can be obtained by ether extraction and flash chromatography purification^[13-14]. The structure of HAS was shown in Fig. 1A.

In ancient China, ZBM was recorded as a condiment food due to its unique flavor, and it became popular in East Asia for hundreds of years. Meanwhile, ZBM was recorded as an herbal medicine for treating various diseases with the functions of “warming energizer and relieving pain, and destroying parasites and relieving itching”^[15-16]. With the deepening understanding of ZBM, this herbal medicine has also been found to have beneficial for improving the learning and memory abilities^[17]. Through the water maze experiment, we have preliminarily screened out the potential active components with the ability to improve learning and memory, and further obtained the active ingredients in its components. The results showed that ethanol extract improved learning and memory ability better than that of water extract and ethyl acetate extract (Fig. S1). At the same time, high performance liquid chromatography (HPLC) was used to detect the content of HAS in ethanol extract of ZBM. It was found that HAS accounted for $(76.97 \pm 0.82)\%$ of the total amide (Fig. S2). Consequently, we speculated that HAS may be a potential natural compound for treating AD. This hypothesis has been further confirmed in a series of subsequent studies.

In the initial study, it was shown that HAS could cause calcium influx in sensory neurons by activating transient receptor potential (TRP) V1 and TRPA1^[18]. Subsequently, the researchers also found that HAS activates neurons by stimulating large-diameter myelinated neurons that express the neurotrophin receptor through a unique mechanism^[14]. It is confirmed that HAS possesses an effect on neurons, but the relationship between this effect and AD is still unknown. Then, in scopolamine-induced mouse models, HAS could protect nerve cells by activating the cholinergic system and cAMP response element-binding protein (CREB)/BDNF signaling pathway, thereby improving the learning and memory ability of mice^[19]. In a

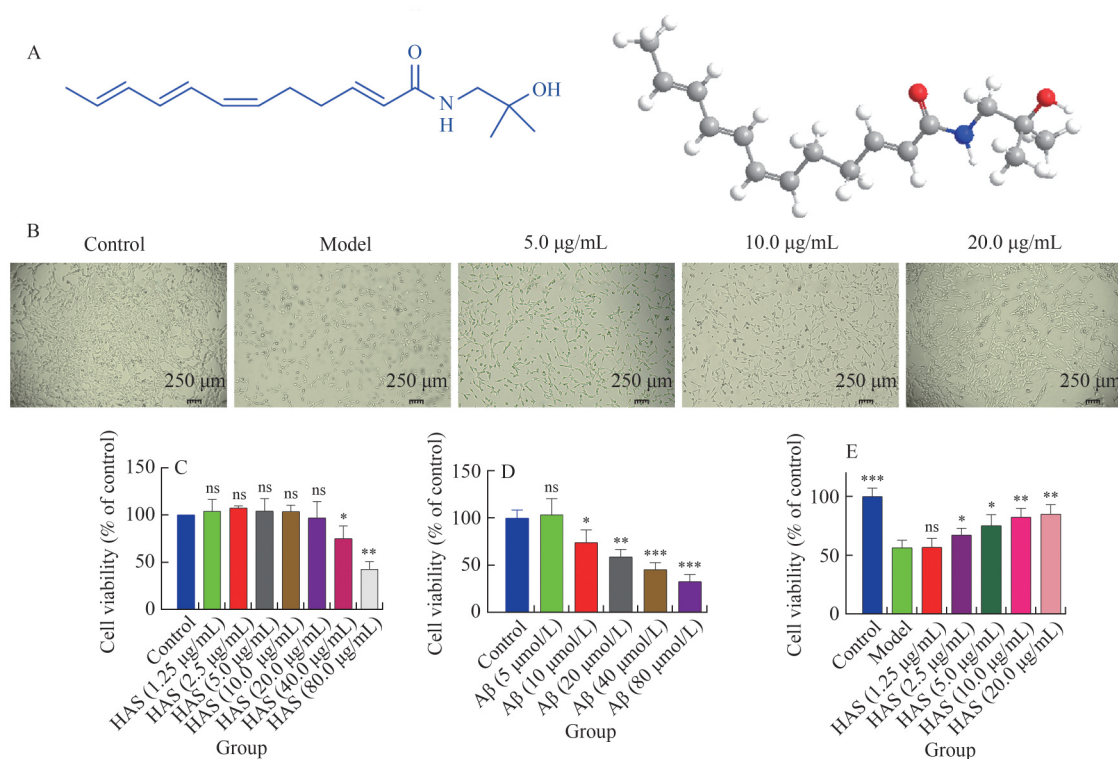


Fig. 1 Protective effects of HAS on the cell viability. (A) The structure of HAS. (B) The represented cell morphology of HT22 cells with HAS treatment. (C) Toxic effects of HAS on HT22 cells. (D) Toxic effects of $A\beta_{1-42}$ on HT22 cells. (E) Protective effects of HAS on the cell viability of $A\beta_{1-42}$ -stimulated HT22 cells. The values were represented as the mean $\pm$ SD ($n = 4$). ns represents $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the model group.

mouse model co-induced by *D*-galactose (*D*-gal) and AlCl_3 , HAS could reduce pathological damage of brain tissue and improve the cognitive ability of mice by activating the nuclear factor erythropoietin-2-related factor 2/heme oxygenase 1 (Nrf2/HO-1) signaling pathway^[20]. However, mice induced by scopolamine showed transient learning and memory deficits, lacking typical pathological changes such as A β deposition and Tau phosphorylation. Although the mouse model co-induced by *D*-gal and AlCl_3 can cause cholinergic system dysfunction and damage of hippocampal neurons, it lacks specificity for AD^[21]. In addition, the detection of typical pathological manifestations of AD is lacking in their experiments. *In vitro* experiments, the researchers selected the rat phochromocytoma cell (PC12) as the research object. It was found that HAS could inhibit apoptosis by relieving oxidative stress in PC12 cells induced by H_2O_2 or $\text{A}\beta_{1-42}$ ^[22-23]. These results suggested that HAS is a potential effective natural agent for AD. In our experiments, we selected senescence-accelerated mouse prone 8 (SAMP8) mice as the *in vivo* AD model. It is well known that the morphological and structural lesions of the brain tissue in SAMP mice are similar to the clinical characteristics of AD^[21]. SAMP8 mice can also show pathological features such as A β deposition and Tau protein hyperphosphorylation, which means that it is an ideal AD mouse model^[21]. *In vitro* experiments, we selected HT22 cells derived from mouse neurons to simulate the nerve cell damage that may occur during the development of AD, thus better revealing the protective effect of HAS. HT22 cells are immortalized neuronal cells. It has characteristics of mature hippocampal neurons, such as neurite outgrowth, expression of functional cholinergic markers and receptors, and is a recognized cell for studying AD. Besides, it was proven by our research that HAS can affect the intestinal microflora in obese mice and improve the diversity of the intestinal microflora in mice as well^[16]. Therefore, we hypothesize that HAS can improve metabolism profile in AD mice by regulating gut microbes, thereby protecting nerve cells.

2. Materials and methods

2.1 Drugs and reagents

HAS (98% purity) was supplied by the PUSH Bio-Technology (Chengdu, China). Donepezil was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Fetal bovine serum (FBS) was obtained from TransGen Biotech (Beijing, China). Dulbecco's modified eagle medium (DMEM), phosphate buffered saline (PBS) and 0.25% trypsin-EDTA (1 $\times$) were purchased from Gibco Co. (Grand Island, NY, USA). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay kit was obtained from 4A Biotech., Ltd. (Beijing, China). The acridine orange/ethidium bromide (AO/EB) assay kit was purchased from Yuanye Bio-Technology Co. (Shanghai, China). The JC-1, rhodamine 123 and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from UELandy Inc (Suzhou, China). The cell counting kit (CCK-8), MitoSOX Red Mitochondrial Superoxide Indicator was purchased from Yeasen Bio-Technology Co. (Shanghai, China). The Mito-Tracker Green was obtained from Beyotime Biotech (Shanghai, China). The assay kits for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from the Michy Biology (Suzhou, China).

2.2 Cell culture

The HT22 cells were purchased from Wuhan Pu-nuo-sai Life Technology Co., Ltd. (Wuhan, China). HT22 cells were cultured in DMEM containing 1% streptomycin (100 $\mu\text{g/mL}$)-penicillin (100 U/mL) and 10% FBS (*V/V*) at 37 °C in a humidified atmosphere of 5% CO_2 . Fresh medium should be replaced every 2 days. At the same time, when the cells reached the exponential growth phase, they should be passaged.

2.3 Determination of cell viability

Cell viability was measured by CCK-8. First, HT22 cells were inoculated into 96-well plates, so that each well contained 100 μL cell suspension with a density of 1×10^5 cells/mL. After 24 h, HT22 cells were attached to the culture plate, and different concentrations of HAS (1.25, 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 $\mu\text{g/mL}$) were added and co-incubated with cells for 24 h. Finally, DMEM containing 10 μL CCK-8 were added and the optical density (OD) at 450 nm was measured. Obtained OD values were used for calculation of cell viability in % of control. In order to select the optimal concentration of $\text{A}\beta_{1-42}$, 5, 10, 20, 40 and 80 $\mu\text{mol/L}$ $\text{A}\beta_{1-42}$ were co-incubated with HT22 cells for 24 h, and the cell viability was detected by CCK-8. After selecting the optimal concentration, we incubated 20 $\mu\text{mol/L}$ $\text{A}\beta_{1-42}$ with HT22 cells for 24 h. Subsequently, the safe dose of HAS was added to incubation for 4 h, and CCK-8 was used to detect OD value. When examining the protective effect of metabolites on $\text{A}\beta_{1-42}$ -induced HT22 cells, the procedure is the same as above. Each experiment in this section was independently repeated 4 times.

2.4 AO/EB staining

HT22 cells were inoculated in confocal plates and treated with the selected $\text{A}\beta_{1-42}$ and HAS concentrations described above. Equal volumes of AO and EB solutions were added to cover the cell surface for 5 min in a dark place. Each experiment in this section was independently repeated 3 times. The images were randomly collected under a laser confocal microscope (Leica, TSC SP8 STED).

2.5 Annexin V-FITC/PI apoptosis assay by flow cytometer

HT22 cells were inoculated into 6-well plates, so that each well contained 2 mL cell suspension with a density of 1×10^5 cells/mL. After HT22 cells were treated as described above, Annexin V-FITC and PI staining was performed according to the manufacturer's instructions. Each experiment in this section was independently repeated 3 times. The flow cytometer (BD Biosciences, FACSCanto II) was used to detect the fluorescence, while the result was processed using FlowJo (version 10.8.1).

2.6 Assessment of mitochondrial membrane potential (MMP)

The effect of HAS on MMP of HT22 cells induced by $\text{A}\beta_{1-42}$ was detected by JC-1 and rhodamine 123. Similarly, HT22 cells were inoculated in confocal plates and pretreated as described above. The JC-1 staining solution (10 $\mu\text{g/mL}$) was added and incubated at 37 °C for 30 min in the dark. Each experiment in this section was

independently repeated 3 times. Images were randomly collected under laser confocal microscope. At the same time, HT22 cells were stained by 1 $\mu\text{g/mL}$ rhodamine 123 at 37 °C for 20 min. The fluorescence intensity of the cells was measured by FACSCanto II flow cytometer, while the result was processed using FlowJo (version 10.8.1).

2.7 Detection of mitochondrial reactive oxygen species (ROS) accumulation

In this part, we used DCFH-DA and MitoSOX Red Mitochondrial Superoxide Indicator to detect intracellular and mitochondrial ROS content. In brief, after the above operation on HT22 cells, DCFH-DA (10 $\mu\text{mol/L}$) was added and incubated at 37 °C for 10 min in the dark. The unbound probe was cleaned off with PBS and the cells were detected by flow cytometer. To detect the mitochondria ROS, Mito-Tracker Green (0.1 $\mu\text{mol/L}$) and MitoSOX Red (5 $\mu\text{mol/L}$) Mitochondrial Superoxide Indicator were added, and incubated at 37 °C for 15 min in dark. After 3 PBS washes, a fluorescent sealant containing DAPI was added, and images were randomly collected under a laser confocal microscope. Each experiment in this section was independently repeated 3 times.

2.8 Detection of CAT, GSH-Px, SOD and MDA activities

The commercial assay kits were used to detect CAT, GSH-Px, SOD and MDA according to the manufacturer's instructions. In brief, HT22 cells were incubated in 6-well plates and given A β_{1-42} or different concentrations of HAS as described above. The washed HT22 cells were lysed using lysis buffer and the total protein was collected. Finally, MDA, SOD, GSH-Px and CAT were detected according to manufacturer's instructions. Each experiment in this section was independently repeated 3 times.

2.9 Animal administration and Morris water maze (MWM) tests

In this experiment, male mice aged 8 months were used as experimental objects. Among them, SAMP8 mice were selected as model group, and the senescence-accelerated mouse resistant 1 (SAMR1) mice were selected as control group. All mice were purchased from Sibeifu Experimental Animal Co. (Beijing, China) and housed under suitable temperature ((22 $\pm$ 2) °C) and humidity ((65 $\pm$ 5)%) in the animal observation room with free access to water and food. The mice were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine. The experimental protocols were approved by the Institutional Ethics Committee of Chengdu University of TCM (approval No. 2020-27). The intragastric dose of HAS was referred to our previous experiment. SAMP8 mice were randomly divided into 5 groups ($n = 8$ in each group): model group (normal saline containing 2% DMSO), positive group (5.0 mg/kg donepezil) and HAS groups (1.25, 2.50, and 5.00 mg/kg HAS). HAS and donepezil were dissolved in normal saline containing 2% DMSO for 60 days intragastric administration. The MWM test was performed after continuous drug treatment for 6 days. We performed MWM test as previously published protocols^[24].

2.10 Histopathological examination

After MWM test, all mice were anesthetized with isoflurane and received intracardial perfusion. The brain tissues were fixed in 4% paraformaldehyde. After a series standard processing, the brain tissues were embedded in paraffin, and subsequently were cut into 5 μm thick sections and stained by H&E and Nissl, respectively^[25-26]. The neuronal structure and integrity in the hippocampus were observed by microscope, and image acquisition was randomized. Each experiment in this section was independently repeated 5 times.

2.11 16S rRNA gene sequencing

Mice feces samples were collected and the DNA of each sample was extracted by the fecal DNA extraction kit. Use 1% agarose gel electrophoresis to detect DNA purity and concentration, followed by dilution of the sample to 1 ng/ μL . The V4 region of the 16S rRNA gene was used for PCR amplification with primer sequences: 515F (5'-GTGCCAGCMGCCGCGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Quantified all PCR products and mixed them in equal amounts. Library construction was carried out using the NEB Next® Ultr™ II FS DNA PCR-free Library Prep Kit. Sequencing was performed using Illumina NovaSeq PE250 platform.

2.12 Metabolomics analysis

Mouse serum metabolites were detected using LC-MS/MS technology at Novogene (Beijing, China). UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q Exactive™ HF-X mass spectrometer (Thermo Fisher, Germany). Samples were injected onto a Hypersil Gold column (100 mm $\times$ 2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mmol/L ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2%–85% B, 3 min; 85%–100% B, 10 min; 100%–2% B, 10.1 min; 2% B, 12 min. Q Exactive™ HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.5 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 psi and aux gas flow rate of 10 L/min, S-lens RF level of 60, Aux gas heater temperature of 350 °C.

The raw data generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5×10^{-6} ; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (<https://www.mzcloud.org/>), mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. After the identification and quantification of metabolites, through principal component analysis (PCA), partial least squares discrimination analysis (PLS-DA), differential metabolite screening and enrichment analysis, etc., to obtain the metabolic effects of HAS on AD mice.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA). The statistical significance between different groups was determined using one-way or two-way ANOVA, $P < 0.05$ was considered statistically significant.

3. Results

3.1 HAS enhanced cell proliferation activity in $A\beta_{1-42}$ -induced HT22 cells

The results were shown in Fig. 1. After incubating with 1.25–20.00 $\mu\text{g/mL}$ HAS for 24 h, the cell proliferation activity of HT22 cells were close to that of control group, and there was no significant change ($P > 0.05$), indicating that HAS in this concentration range had no obvious toxicity to HT22 cells. However, when the HAS concentration was increased to 40 or 80 $\mu\text{g/mL}$, the cell proliferation activity decreased to $(73.00 \pm 3.30)\%$ or $(40.71 \pm 5.97)\%$ ($P < 0.05$ or $P < 0.01$). Therefore, in the follow-up experiment, we consider choosing a safe dose range of 1.25–20.00 $\mu\text{g/mL}$ for HAS. When choosing the optimal concentration of $A\beta_{1-42}$, the results suggested that 10–80 $\mu\text{mol/L}$ $A\beta_{1-42}$ could reduce the cell proliferation activity to vary degrees, while 20 $\mu\text{mol/L}$ $A\beta_{1-42}$ could reduce the cell proliferation activity to $(58.84 \pm 6.79)\%$, and 40 $\mu\text{mol/L}$ $A\beta_{1-42}$ could reduce the cell proliferation activity to $(45.36 \pm 6.30)\%$ ($P < 0.001$). When the cell proliferation activity is too low, the cell survival is difficult, which is not conducive to the follow-up experiment^[27]. Therefore, 20.00 $\mu\text{mol/L}$ $A\beta_{1-42}$ was selected as the optimal concentration of $A\beta_{1-42}$ in subsequent experiments. After using 1.25–20.00 $\mu\text{g/mL}$ HAS to explore its protective effect on $A\beta_{1-42}$ -induced HT22 cells, the results suggested that 2.50–20.00 $\mu\text{g/mL}$ HAS could significantly improve the cell proliferation activity, while 5, 10 and 20 $\mu\text{g/mL}$ HAS had better effect. In the model group, we can observe more morphological changes of HT22 cells, such as cell morphology shrinkage, cell size

reduction, and the reduced number of cell adherents. To sum up, 20 $\mu\text{mol/L}$ $A\beta_{1-42}$ would be selected as the modelling concentration, while 5, 10 and 20 $\mu\text{g/mL}$ HAS would be selected as the therapeutic concentration for administration in subsequent cell experiments.

3.2 HAS inhibited apoptosis in $A\beta_{1-42}$ -induced HT22 cells

In our experiments, we performed qualitative and quantitative analysis of apoptosis by AO/EB staining and Annexin V-FITC/PI staining, respectively. In AO/EB staining, when apoptosis occurs, EB will enter the cell and embed nuclear DNA, causing detection of red fluorescence. The results indicated that the intensity of red fluorescence in HT22 cells increased after incubation with $A\beta_{1-42}$, while the proportion of green fluorescence decreased. After treatment with 5–20 $\mu\text{g/mL}$ HAS, the above situation was reversed, preliminarily indicating that HAS inhibited $A\beta_{1-42}$ -induced apoptosis (Fig. 2A). Apoptosis was quantified by flow cytometry in Annexin V-FITC/PI staining. It was shown that apoptosis increased to $(36.49 \pm 2.88)\%$ after $A\beta_{1-42}$ stimulation, and decreased to $(27.75 \pm 1.34)\%$ ($P < 0.05$), $(24.90 \pm 1.02)\%$ ($P < 0.01$) and $(16.29 \pm 0.94)\%$ ($P < 0.01$) after 5–20 $\mu\text{g/mL}$, respectively (Fig. 2B). At the same time, as an early event of apoptosis, the decrease of MMP will lead to a series of apoptotic cascades such as the opening of permeability conversion pore. We used JC-1 to detect MMP. When MMP was higher, the ratio of red fluorescence intensity was higher. After $A\beta_{1-42}$ stimulation, the red fluorescence ratio decreased significantly, indicating that MMP decreased in HT22 cells (Fig. 3A). However, MMP was elevated after treatment with different concentrations of HAS ($P < 0.05$ and $P < 0.01$). The same result was shown in rhodamine 123 (Fig. 3B). When MMP decreases, rhodamine 123 will release mitochondria, resulting in increased fluorescence intensity. In our experiment, the results showed that the fluorescence intensity increased after $A\beta_{1-42}$ stimulation, while the fluorescence intensity was weakened by different concentrations of HAS ($P < 0.01$).

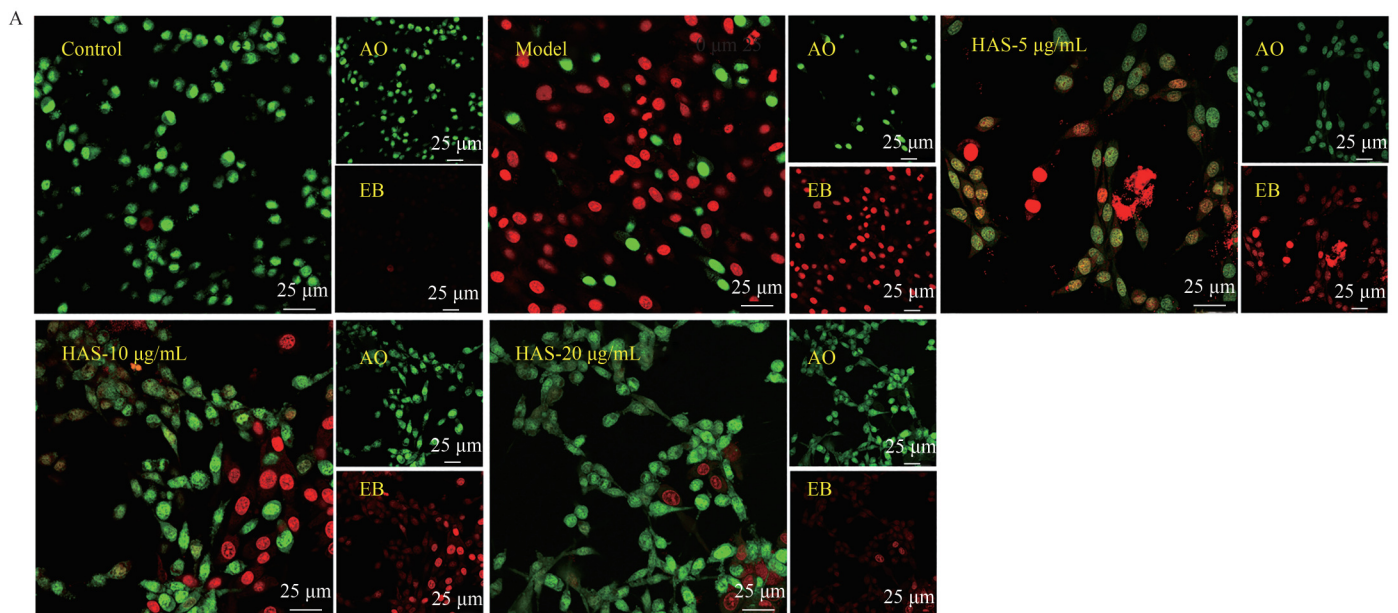


Fig. 2 HAS inhibited apoptosis in $A\beta_{1-42}$ -induced HT22 cells. (A) AO/EB staining. (B) Apoptotic assay by flow cytometry. Data are expressed as means $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. the model group.

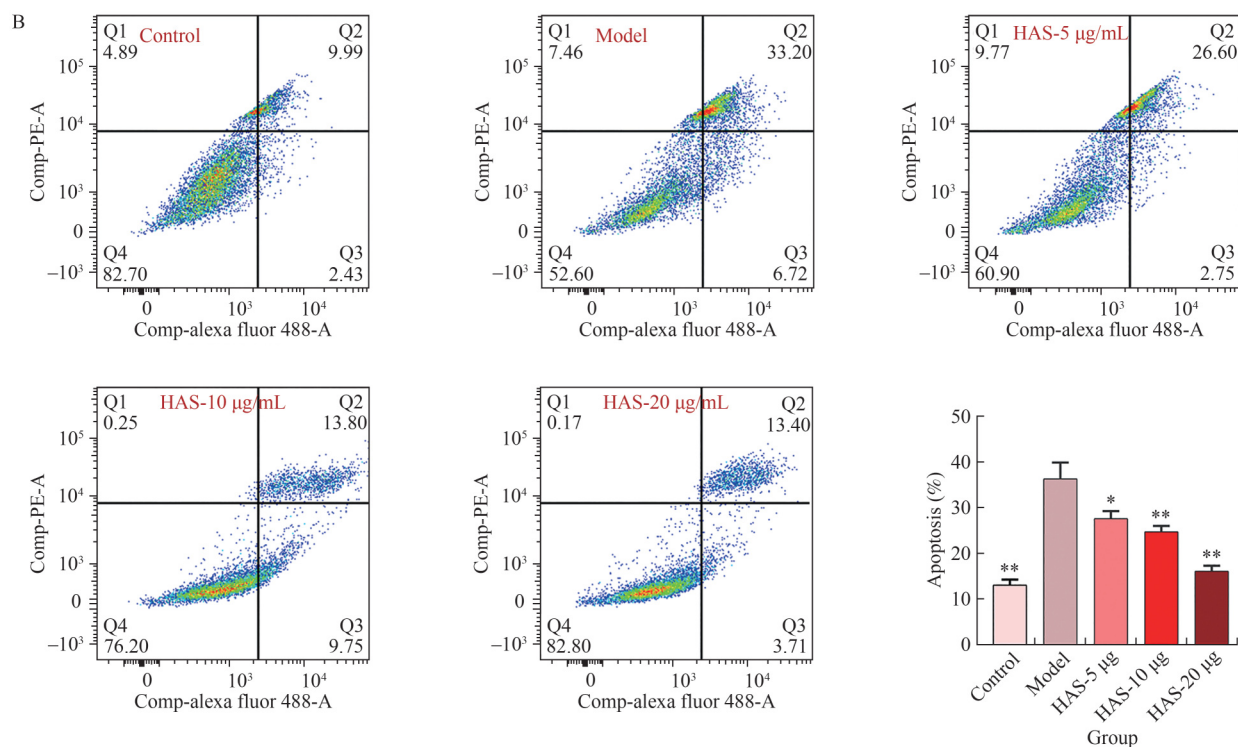


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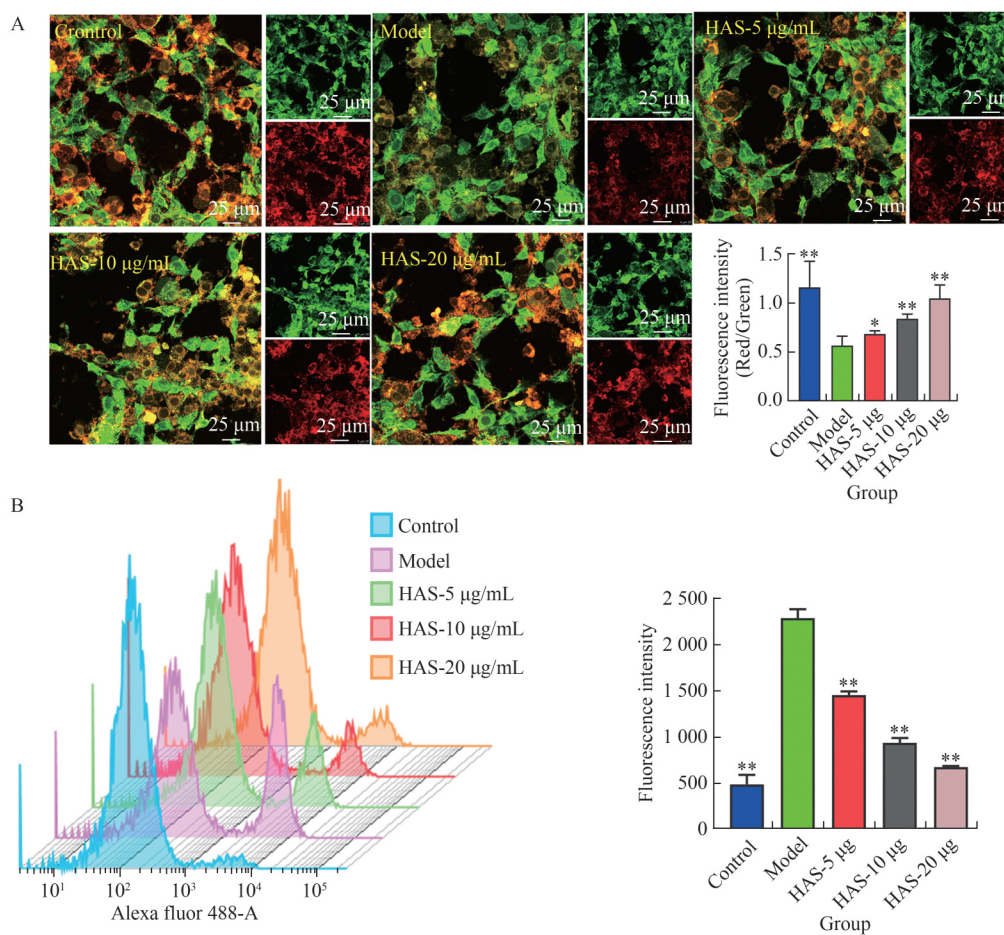


Fig. 3 HAS increased mitochondrial membrane potential in HT22 cells after incubation with $\text{A}\beta_{1-42}$. (A) JC-1 staining. (B) Rhodamine 123 staining detected by flow cytometry. Data were expressed as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. the model group.

3.3 HAS inhibited oxidative stress in $A\beta_{1-42}$ -induced HT22 cells

It is well known that intracellular oxidative stress is an important pathway leading to apoptosis, and the accumulation of excessive ROS

is the main cause of cellular oxidative stress. Therefore, intracellular ROS was detected in experiments. As shown in Fig. 4A, ROS level in HT22 cells increased significantly after $A\beta_{1-42}$ stimulation ($P < 0.01$). In addition, mitochondrial respiratory chain is not only the main site

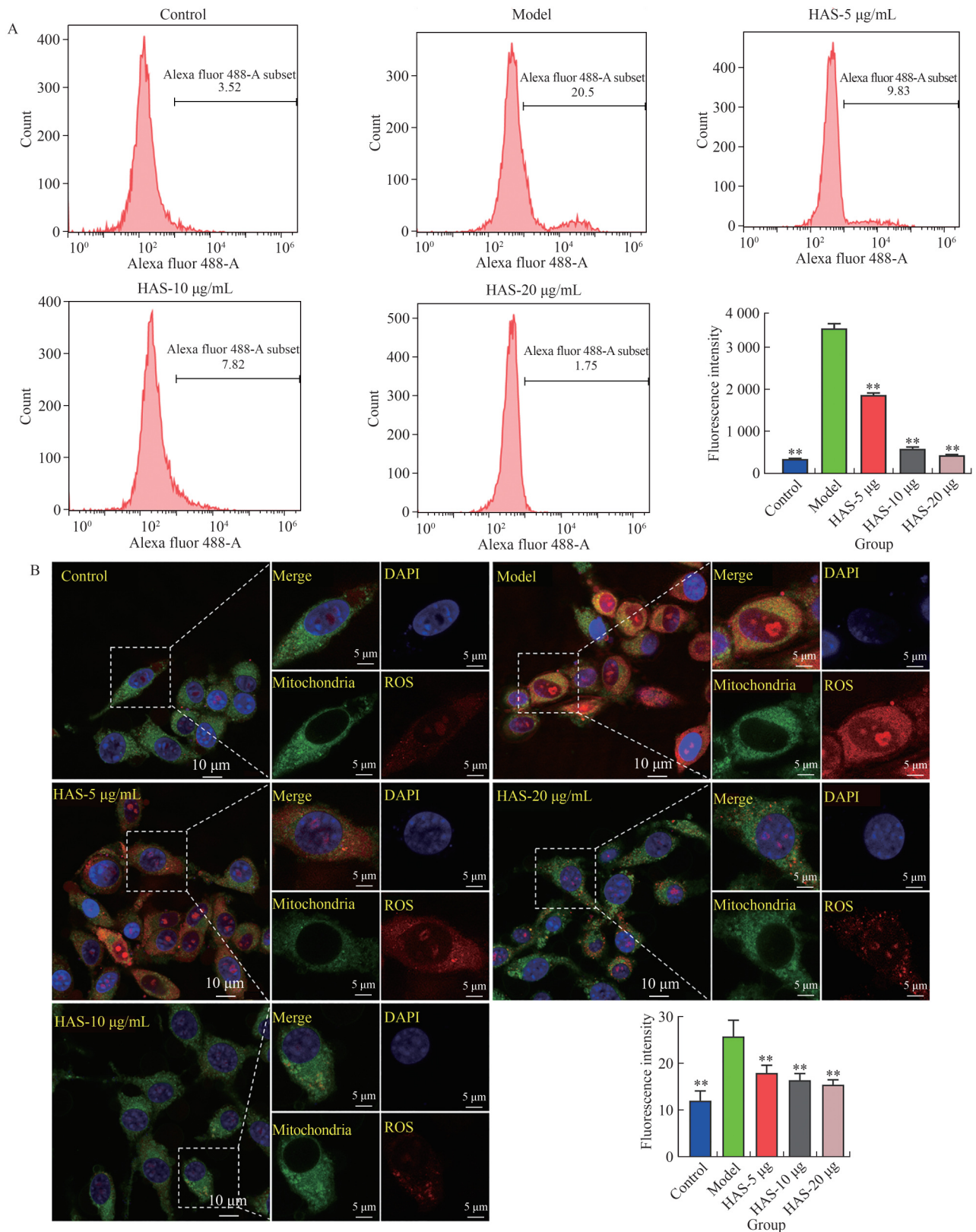


Fig. 4 HAS inhibited oxidative stress in $A\beta_{1-42}$ -induced HT22 cells. (A) The ROS level in HT22 cells were detected by flow cytometry. (B) The mitochondrial ROS staining. (C) The levels of CAT, GSH-Px, SOD and MDA. Data were expressed as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. model group.

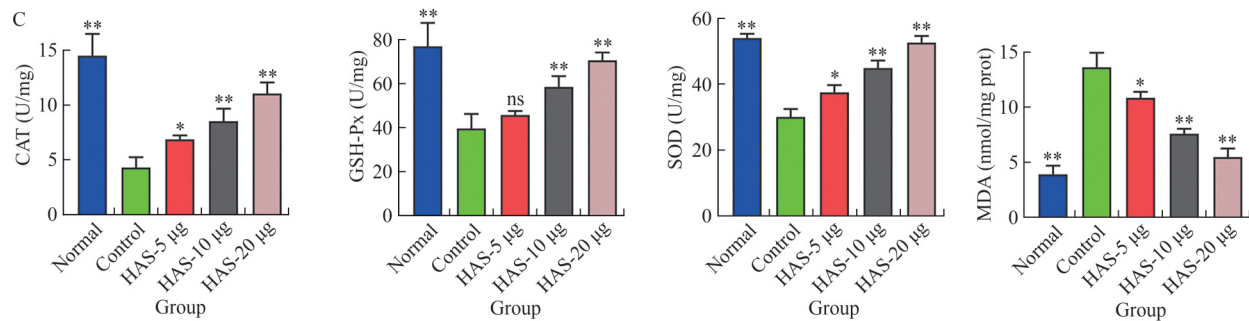


Fig. 4 (Continued)

of energy generation, but also the main pathway of ROS generation. Therefore, mitochondrial ROS is often used as an important indicator of oxidative stress. In our results, ROS content in mitochondria increased significantly after $\text{A}\beta_{1-42}$ stimulation ($P < 0.01$) (Fig. 4B). Fortunately, when 5–20 $\mu\text{g/mL}$ HAS were administered, the intracellular and mitochondrial ROS content was decreased ($P < 0.01$).

CAT, GSH-Px and SOD are several important antioxidant enzymes in the body, and they work together to remove oxygen free radicals. Meanwhile, MDA, as a product of lipid peroxides, can be used to indirectly evaluate the severity of free radical attack on cells. In Fig. 4C, $\text{A}\beta_{1-42}$ decreased the activities of CAT, GSH-Px and SOD, and produced more MDA ($P < 0.01$). However, HAS could reverse the above situation to varying degrees, increasing the activities of CAT, GSH-Px and SOD while reducing the content of MDA.

Based on the above results, we can conclude that HAS may maintain the intracellular redox balance by restoring the activities of

CAT, GSH-Px and SOD, avoid excessive ROS accumulation, and further inhibit the occurrence of apoptosis.

3.4 HAS improved the learning and memory ability of SAMP8 mice

The MWM test is a well-established test used to detect spatial learning and long-term memory in animals. As expected, there was no significant change in latency time in the Model group during the MWM experiment. The escape latency time in Control group and HAS group decreased in these days. On day 5 and day 6, the HAS-treated SAMP8 mice found the platform more quickly than SAMP8 mice, showing stronger learning and memory ability (Figs. 5A and B). At the same time, mice in the Control group and the HAS group stayed in the target quadrant significantly longer than those in the Model group ($P < 0.01$), and the platform crossing times were significantly more than those in the Model group (Figs. 5C and D).

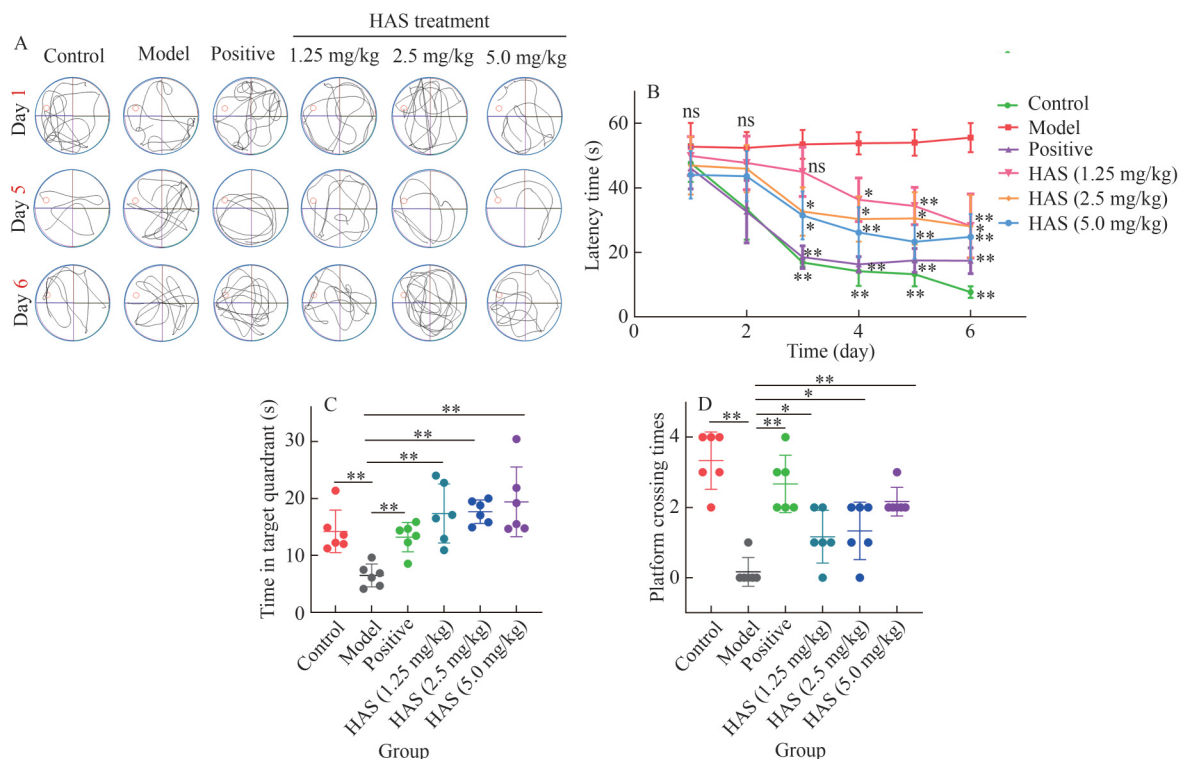


Fig. 5 HAS attenuated learning and memory deficits and protected hippocampal neuron. (A) Track diagram of SAMP8 mouse in the experiment. (B) The mean escape latency time in Morris water maze test. (C) The residence time spent in the target quadrant. (D) The number of crossing platforms. * $P < 0.05$, ** $P < 0.01$, ns $P > 0.05$, vs. the model group.

3.5 HAS reduced hippocampus neuronal damage in SAMP8 mice

Neurons in the hippocampus, an area associated with learning and memory, are vulnerable to damage that can affect learning and memory. According to the results of HE staining (Fig. 6A), hippocampal neurons in SAMP8 mice were atrophied, with disordered cell arrangement. HAS significantly improved this phenomenon. At the same time, the number of Nissl positive cells in the hippocampus of SAMP8 mice was significantly decreased, while the number of Nissl positive cells was increased by HAS treatment (Figs. 6B and C).

3.6 HAS modulated the intestinal microbiome of SAMP8 mice

Previous studies have shown that the gut microbiome is closely related to the learning and memory ability of AD mice. We tested mice faeces with 16S rRNA gene sequencing. The experimental results suggested that the HAS group shared 644 operational taxonomic units (OTUs) with Control group and 491 OTUs with Model group, while the unique OTUs included in Control, Model, and HAS were 936, 258, and 667, respectively (Fig. 7A). In this part, we can obviously observe that OTUs in HAS group were increased compared with that of Model group, which preliminarily suggests that

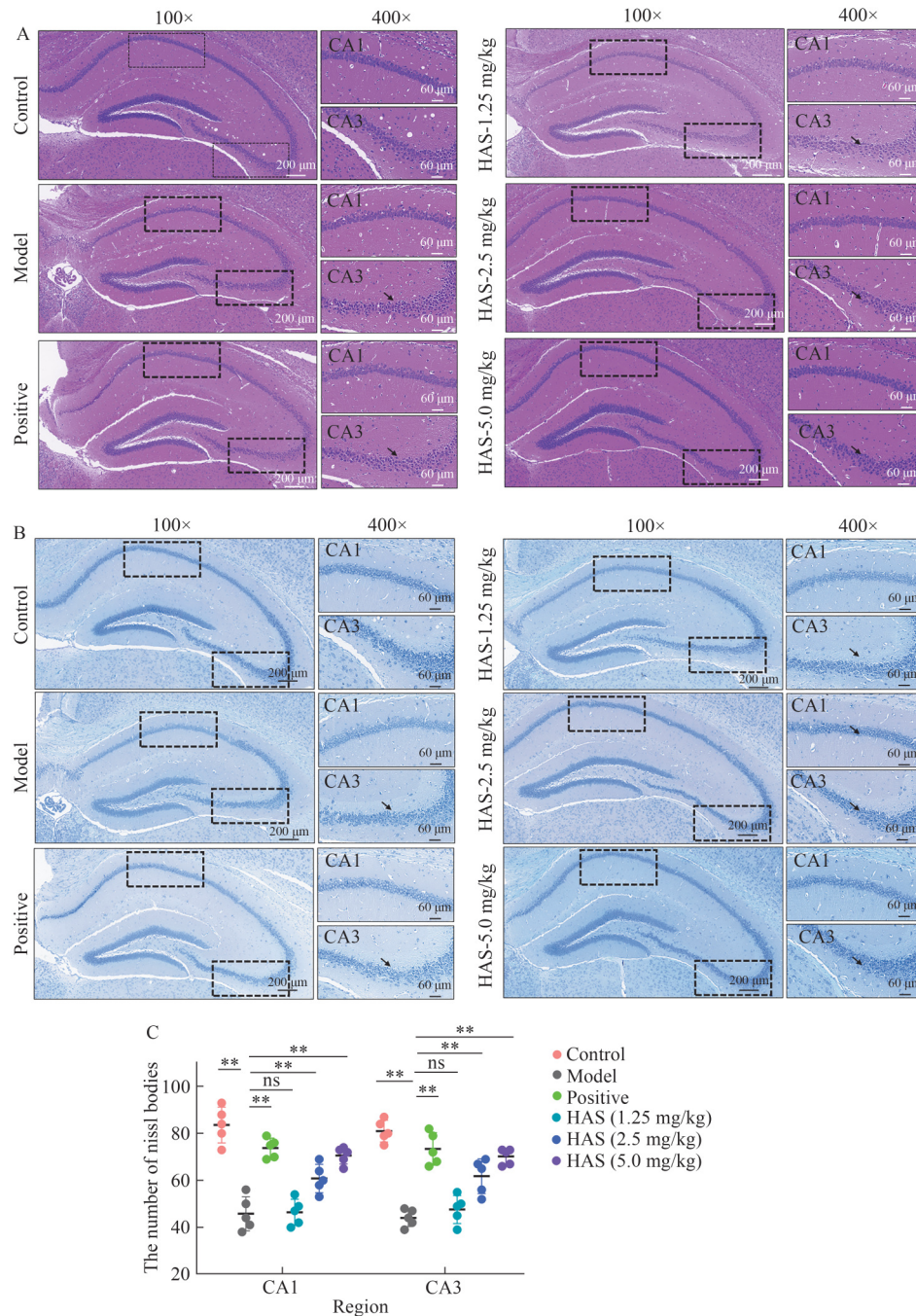


Fig. 6 HAS protected hippocampal neuron in SAMP8 mice. (A) Representative photomicrographs of HE staining in the hippocampal CA1 and CA3 regions. (B) Representative photomicrographs of Nissl staining in the hippocampal CA1 and CA3 regions. (C) The number of Nissl bodies. ** $P < 0.01$, ns $P > 0.05$, vs. the model group.

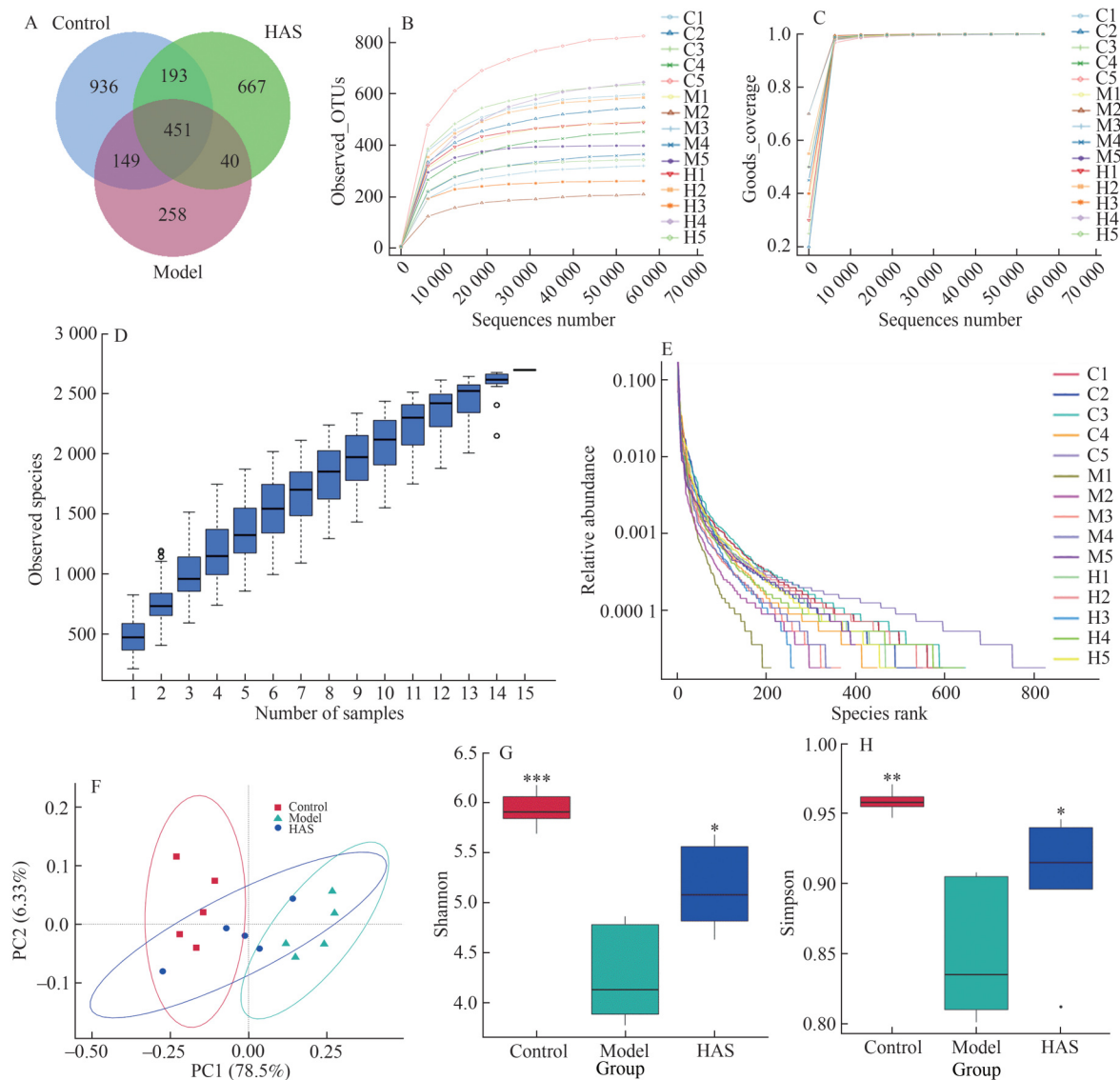


Fig. 7 Relevant sequencing data from mice faeces. (A) Venn diagram of OTUs. (B) Rarefaction curve. (C) Goods_coverage. (D) Species accumulation boxplot. (E) Rank abundance. (F) PCoA using weighted UniFrac. (G) Shannon index. (H) Simpson index. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the model group.

HAS may increase the intestinal flora richness of SAMP8 mice. In order to verify whether the sequencing volume of this experiment is reasonable, rarefaction curve, Goods_coverage and species accumulation boxplot were calculated. The results are shown in Figs. 7B-D. When the sample sequencing data reached a certain amount, the curve tended to be stable, indicating that the depth and data amount of the sequencing were set reasonably (Figs. 7B and C). Fig. 7D reflected the rate of new species under continuous sampling. The results showed that the position of the box diagram tended to smooth with the increase of sample. It means that under this condition, the intestinal communities will not significantly increase with the increase of the sample, and the sample volume of this experiment is sufficient. In rank abundance, the curve width of samples in Control group and HAS group was wider than that in Model group, indicating higher microbial richness (Fig. 7E). In PCoA result, the intestinal flora in Model group and the Control group were divided into two regions, indicating a difference in microbial composition (Fig. 7F). However, after SAMP8 mice received HAS treatment, the PCoA

diagram shifted to Control group. In the PCoA, the closer the sample distance, the more similar the species composition structure. Therefore, this result also confirmed that HAS improved the microbial community structure in the gut of SAMP8. The Shannon's diversity index and Simpson index can jointly illustrate the diversity of microbial community. As shown in Figs. 7G and H, intestinal microbial diversity of SAMP8 mice decreased, while diversity increased after the administration of HAS. Based on the above results, we can conclude that HAS can improve the diversity and richness of intestinal microorganisms in SAMP8 mice.

We analyzed the intestinal microbiota composition of mice at phylum and genus levels (Fig. 8). At the phylum level, compared with SAMR1 mice, the richness of Bacteroidetes in intestinal microflora of SAMP8 mice decreased obviously, while Firmicutes increased. At the genus level, the *Bacteroides*, *Alistipes*, *Akkermansia* and *Parabacteroides* of SAMP8 mice decreased, while *Enterorhabus* and *Lactobacillus* increased. In contrast, when SAMP8 mice were treated with HAS, all of these conditions were reversed. In addition, during linear discriminant

analysis (LDA) Effect Size (LEfSe) analysis the LDA threshold is set to 2.5. The results showed that p_Firmicutes, f_Lactobacillaceae and g_Lactobacillus were the characteristic markers of the Model group, and f_Bacteroidaceae, g_Bacteroides and g_Parabacteroides were the characteristic markers of the Control group.

3.7 HAS regulated metabolites of SAMP8 mice

OPLS-DA was used to analyze the intergroup metabolic differences between SAMR1 and SAMP8 mice or between HAS + SAMP8 and SAMP8 mice. Figs. 9A-D shows the results. In OPLS-DA diagram,

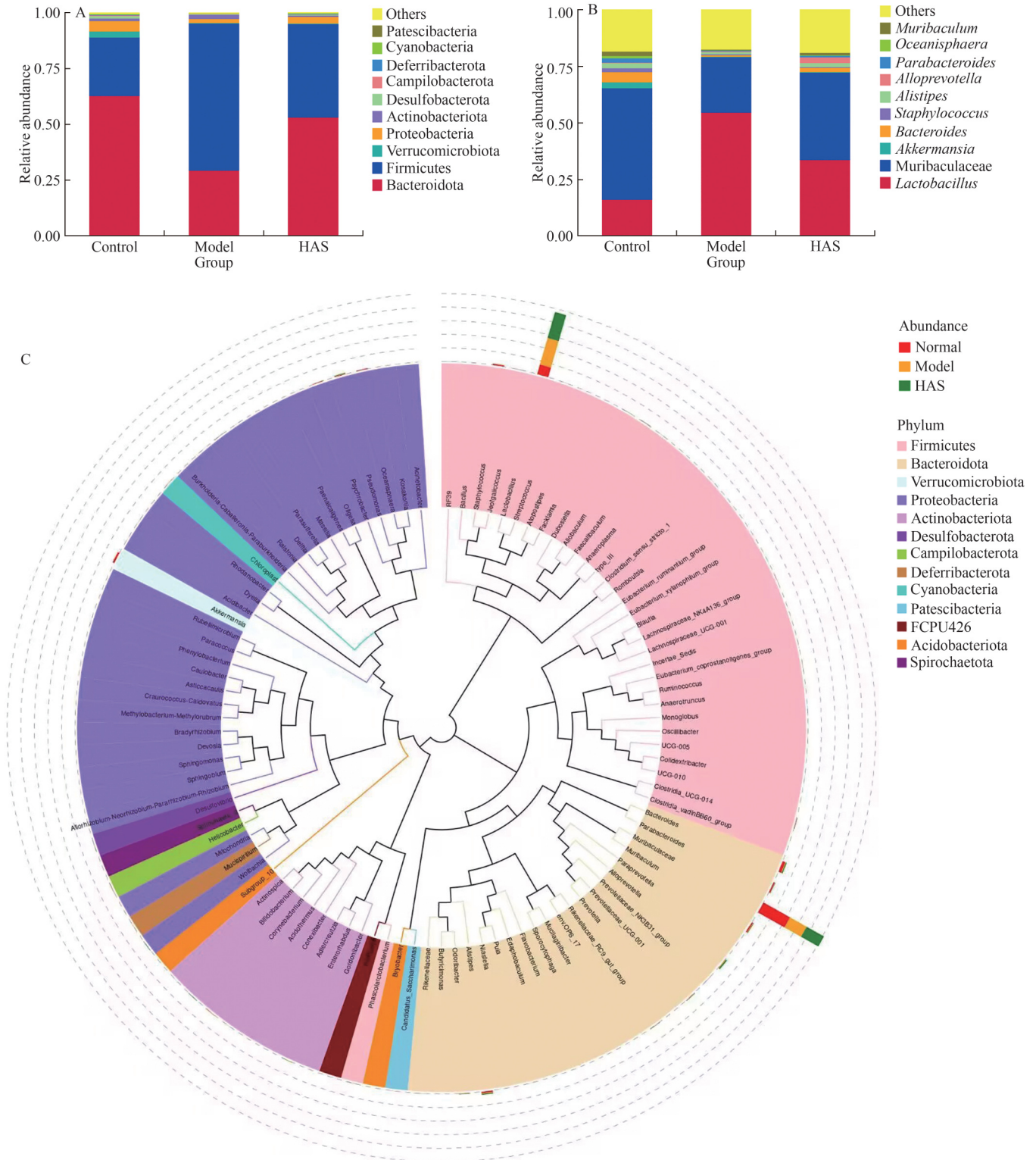


Fig. 8 (A) Intestinal microbiota composition of mice at phylum level. (B) Intestinal microbiota composition of mice at the genus level. (C) Genus horizontal species evolutionary tree. (D) The MetaStat Analysis at phylum level. (E) The MetaStat Analysis at genus level. (F) Clustering heat map of top 20 species abundance at genus level. (G) LEfSe analysis.

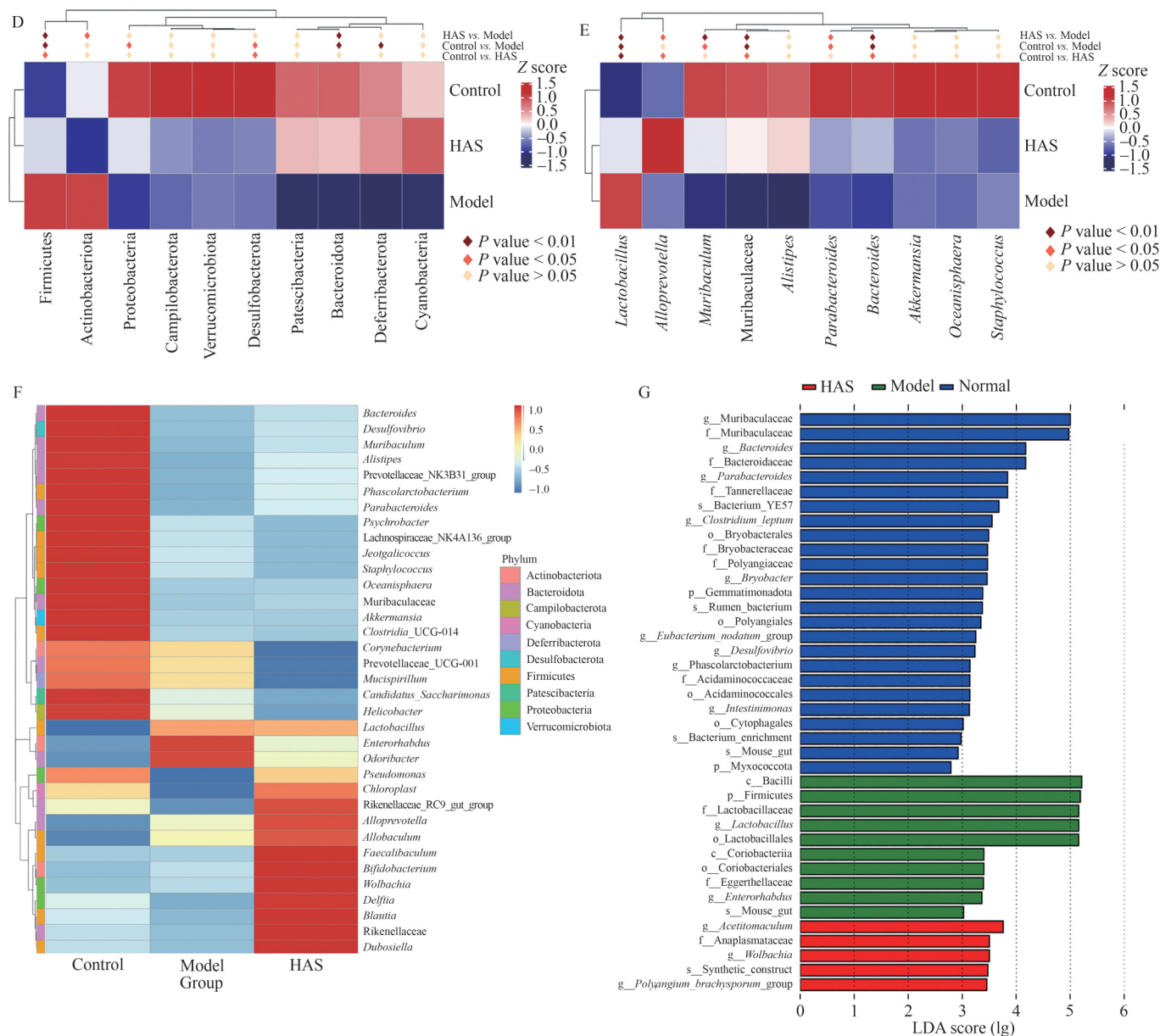


Fig. 8 (Continued)

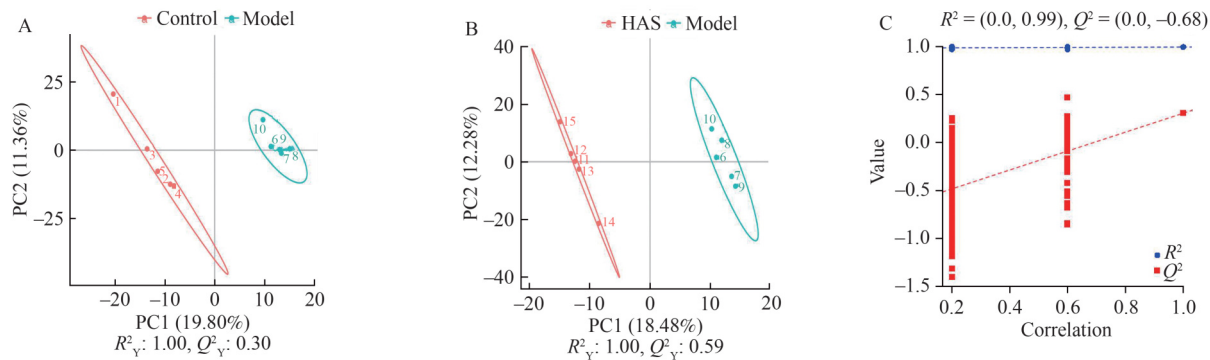


Fig. 9 Variation characteristics of serum metabolites and screening for differential metabolites between SAMR1, SAMP8 and HAS + SAMP8 mice. (A) PLS-DA score plots of the Control and Model group. (B) PLS-DA score plots of the HAS and Model group. (C) PLS-DA statistical validations of the Control and Model group. (D) PLS-DA statistical validations of the HAS and model group. (E) Volcano plot of the control and model group. (F) Volcano plot of the HAS and model group. (G) The top 20 metabolic pathways in the Control and Model group. (H) The top 20 metabolic pathways in the HAS and Model group. (I) Heatmap of 10 differential metabolites.

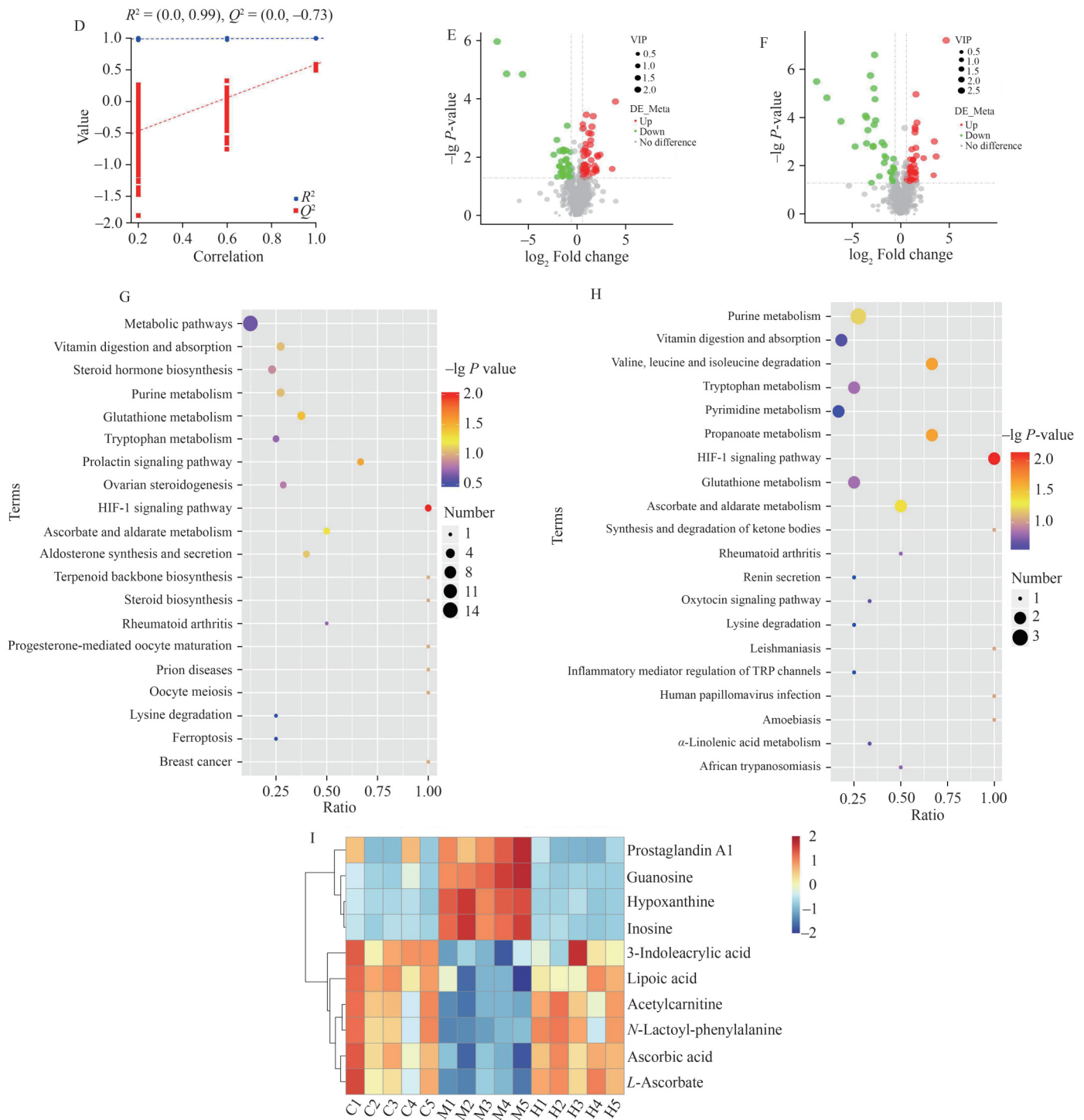


Fig. 9 (Continued)

SAMR1 and SAMP8 mice were completely separated, indicating that there were potential metabolic markers that could be distinguished between SAMR1 and SAMP8 mice. In this model, the values of R^2_Y and Q^2_Y are 1.00 and 0.30, indicating that the model is stable and reliable. Meanwhile, R^2 and Q^2 were 0.99 and -0.68 , indicating that the model was not “overfitted”. In addition, the OPLS-DA of the HAS group and the Model group were also completely separated, and the values of R^2_Y , Q^2_Y , R^2 and Q^2 were 1.00, 0.59, 0.99 and -0.73 , respectively, indicating that this model met the conditions.

Subsequently, with variable importance in the projection (VIP) > 1, fold change (FC) > 1.5, and $P < 0.05$ as the screening condition, the identified metabolites were screened and the volcanic map was drawn. The results were shown in Figs. 9E and F. Compared with SAMP8 mice, 48 differential metabolites were significantly up-regulated and 34 differential metabolites were significantly down-regulated in SAMR1 mice ($P < 0.05$), and these differential metabolites were mainly concentrated in purine metabolism, glutathione metabolism, tryptophan metabolism, hypoxia-inducible factor (HIF)-1 signaling

pathway, ascorbate acid and aldarate metabolism. Notably, compared with the Model group, 49 metabolites were significantly up-regulated and 36 differentially metabolized metabolites were down-regulated in HAS group ($P < 0.05$). The pathways mainly involved pyrimidine metabolism, purine metabolism, glutathione metabolism, tryptophan metabolism, and so on (the top 20 metabolic pathways were shown in Figs. 9G and H). Among them, purine metabolism, glutathione metabolism and tryptophan metabolism are the typical metabolic pathways shared by the 3 groups. Purine metabolism, as an important intermediate link, can affect a variety of metabolic pathways, such as adenosine triphosphate, cyclic adenosine monophosphate, reduced nicotinamide adenine dinucleotide and coenzyme A, and thus affect body homeostasis. At the same time, purine in purine metabolism is an important part of DNA and RNA nucleobase backbone, and its content change will directly affect body homeostasis^[28]. Glutathione, as an active non-protein mercaptan compound widely existing in organisms, is a common antioxidant, and its metabolism will directly affect the antioxidant properties of the body and then affect AD^[29]. In addition, it has been reported that in tryptophan metabolism, tryptophan can metabolize through kynurenine pathway to generate neuroactive molecules (kynurenic acid) and neurotoxic molecules (quinolinic acid), and then affect the central nervous system^[30]. Subsequently, according to the human metabolome database (HMDB), 10 differential metabolites shared by 3 groups, which were closely related to AD, were screened as potential differential biomarkers (Fig. 9I). Compared to the Control and HAS groups, the contents of 3-indoleacrylic acid, acetylcarnitine, ascorbic acid, *L*-ascorbate, lipoic

acid, and *N*-lactoyl-phenylalanine in Model group decreased significantly, while the contents of guanosine, hypoxanthine, inosine and prostaglandin A₁ (PGA1) in Model group increased significantly. The data of ten different metabolites were shown in Table S1.

3.8 Differential biomarkers have protective effects on nerve cells

In the above experiments, we screened for differential biomarkers. Finally, 10 differential biomarkers were selected as typical differential biomarkers. Since 2 differential metabolites, including 3-indoleacrylic acid and *N*-lactoyl-phenylalanine have not been clearly reported, we examined their effects on nerve cells in subsequent experiments using CCK-8 and flow cytometry. The results showed that 1–100 $\mu\text{mol/L}$ 3-indoleacrylic acid had no significant toxicity to HT22 cells (Fig. 10A), while 150 $\mu\text{mol/L}$ 3-indoleacrylic acid could slightly decrease the proliferation activity of HT22 cells (Fig. 10A). In addition, 10–80 $\mu\text{mol/L}$ 3-indoleacrylic acid could significantly improve the cell proliferation activity in $\text{A}\beta_{1-42}$ -induced HT22 cells (Fig. 10B). After the cells were treated with 40 $\mu\text{mol/L}$ 3-indoleacrylic acid and $\text{A}\beta_{1-42}$, the apoptosis of HT22 cells was detected. The results showed that 3-indoleacrylic acid could reduce the apoptosis rate to $(15.4 \pm 0.46)\%$ (Fig. 10C). *N*-Lactoyl-phenylalanine had similar results. That is, 1–10 $\mu\text{mol/L}$ *N*-lactoyl-phenylalanine have no significant effect on HT22 cell proliferation activity, while 20–40 $\mu\text{mol/L}$ *N*-lactoyl-phenylalanine slightly increased $\text{A}\beta_{1-42}$ -induced HT22 cell proliferation activity. At the same time, 20 $\mu\text{mol/L}$ *N*-lactoyl-phenylalanine could reduce apoptosis to $(38.18 \pm 1.26)\%$.

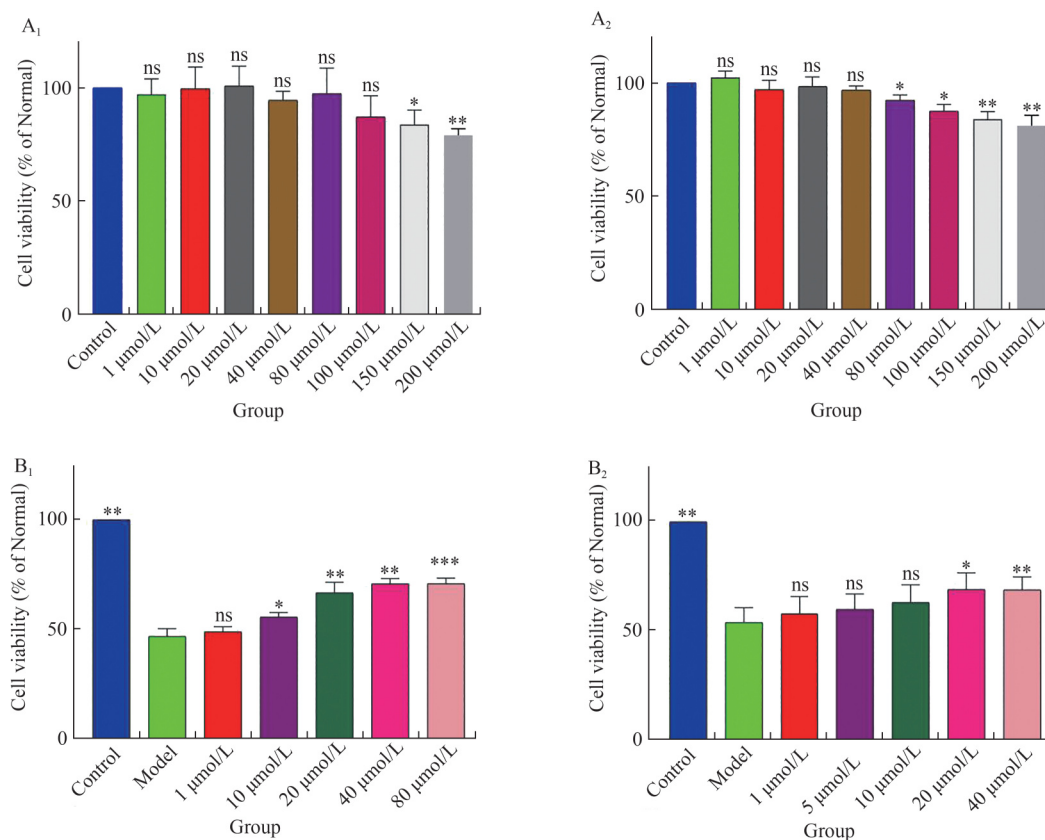


Fig. 10 Effects of differential metabolites on nerve cells. (A) Toxic effects on HT22 cells; (A₁) 3-indoleacrylic acid, (A₂) *N*-lactoyl-phenylalanine. (B) Effects on the cell viability of $\text{A}\beta_{1-42}$ -stimulated HT22 cells; (B₁) 3-indoleacrylic acid, (B₂) *N*-lactoyl-phenylalanine. (C) Effects on the apoptosis rate of $\text{A}\beta_{1-42}$ -stimulated HT22 cells.

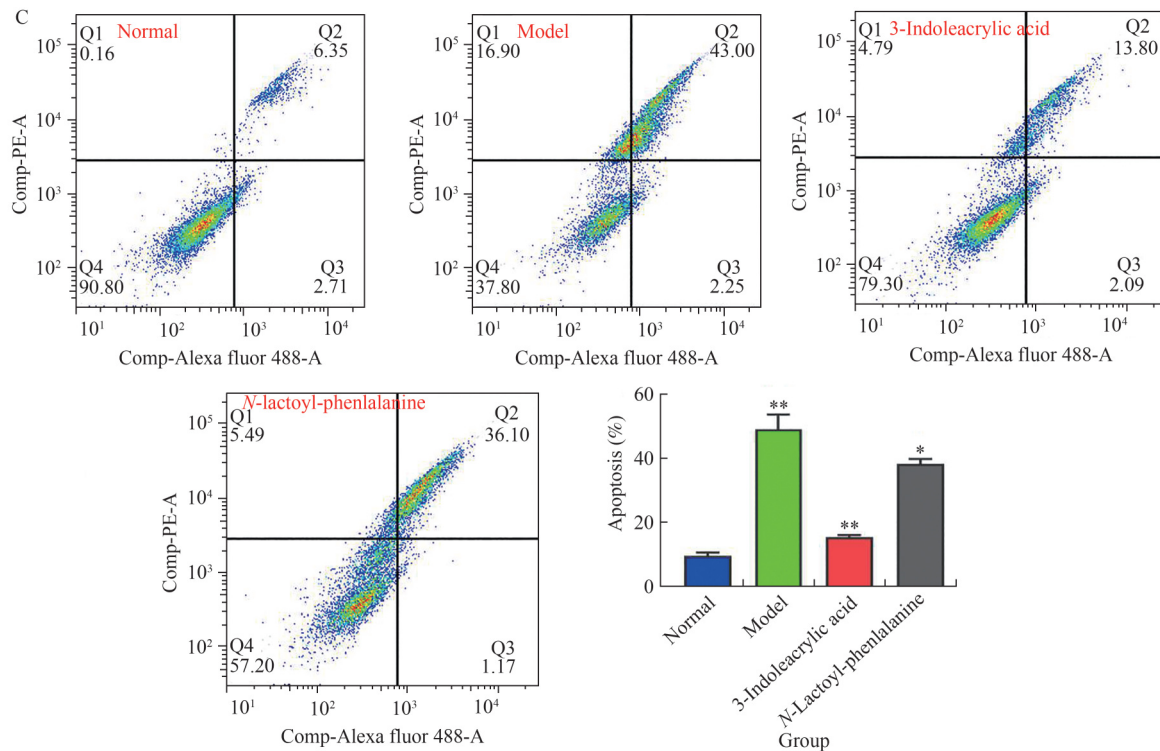


Fig. 10 (Continued)

3.9 Correlation analysis between metabolomics and microbial communities

To further explore the relationship between intestinal microbiota and serum metabolites, Spearman correlation analysis was used to correlate characteristic microbiota at genus level with serum marker differential metabolites. The results are shown in Fig. 11. It is worth noting that 3-indoleacrylic acid was positively correlated with *Parabacteroides* ($P < 0.05$). In addition, PGA1 was also negatively associated with *Bacteroides* ($P < 0.05$) and *Parabacteroides* ($P < 0.01$).

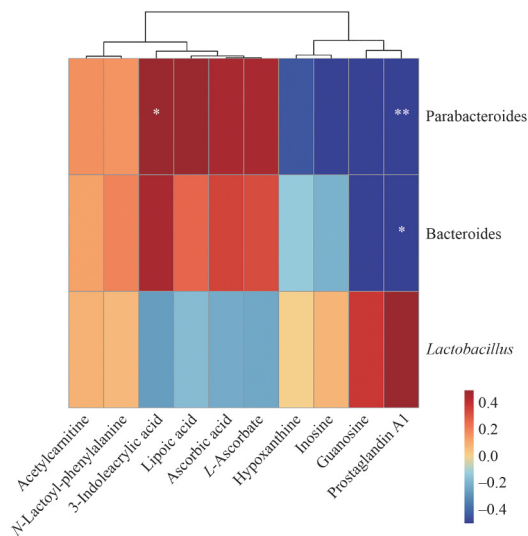


Fig. 11 Correlation analysis of gut microbiota and serum metabolites at the genus level. Blue shows a negative correlation and red shows a positive correlation. * $P < 0.05$, ** $P < 0.01$ vs. the model group.

4. Discussion

As a progressive neurodegenerative disease, AD has an insidious onset. A large number of pathophysiological changes have occurred before the clinical manifestations of AD can be formally observed, among which excessive accumulation of A β and phosphorylation of Tau protein induced neuronal apoptosis is the most typical characteristics. Subsequently, neurons in the cerebral cortex and connections between the temporal lobe and hippocampus are lost, leading to learning and memory impairment^[31]. Existing drugs that can be used to treat AD mainly include acetylcholinesterase inhibitors and *N*-methyl-*D*-aspartate receptor antagonists. Among them, acetylcholinesterase inhibitors represented by donepezil and galantamine can maintain neuronal activity by increasing the content of acetylcholine. However, due to the poor ability to penetrate the blood-brain barrier, their therapeutic ability has certain limitations. At the same time, they can also cause adverse reactions such as nausea, diarrhoea and arrhythmia after long-term use, which further limits their application. Owing to drugs used to treat AD commonly have side effects, researchers have never stopped exploring new AD treatment. Natural products come from a wide range of sources, have excellent performance in anti-inflammatory, anti-apoptotic and other aspects^[32]. As a kind of traditional Chinese medicine, the role of ZBM in the treatment of AD has been paid more and more attention. In our previous experiments, we found that ethanol extract of ZBM had the strongest effect on cognitive ability improvement, and had the highest content of amide compounds. As a unique flavor component, the therapeutic effect of amides in AD has also been studied by researchers. *N*-[2-(3,4-Dimethoxyphenyl)ethyl]-3-phenyl-acrylamide, also known as gx-50, is an amide component in ZBM. Previous studies have shown that gx-50 can inhibit glycogen synthase kinase 3 (GSK-3) activity by promoting protein kinase B (Akt) phosphorylation, restore the transcriptional activity of CREB,

and ultimately promote BDNF expression, thus treating AD^[33]. Qinbunamides B, ZP-amide C and ZP-amide D are isobutyl hydroxylamines that have been isolated from ZBM and identified. In experiments conducted by Tian et al.^[34], all 3 compounds enhanced NGF activity and thereby stimulated the neuronal differentiation of PC12 cells, with potential neuroprotective effects. HAS has the highest content in the total amide of ZBM and contributes greatly to the unique flavor of ZBM. It has been confirmed that HAS could improve learning and memory ability, but the detail mechanisms have not been fully explained. In our experiments, 8-month-old SAMP8 mice took longer time to find the platform in the MWM test than SAMR1 mice. After 5 days of training, they crossed the platform less often than SAMR1 mice and stayed in the target quadrant for less time. The above results suggest that significant learning and memory impairment has emerged in SAMP8 mice. After continuous HAS treatment, the learning and memory ability of SAMP8 mice can be significantly improved. The time to find the platform was shortened, the number of times to pass the platform was increased, and the time spent in the target quadrant was prolonged. Subsequently, the hippocampi of mice brain tissue was examined pathologically. In the hippocampal region of SAMP8 mice brain tissue, nerve cells arrangement were disordered, neuronal atrophy occurred, and the number of Nissl-positive cells was also reduced. After HAS treatment, the morphology of neuronal cells in the hippocampal returned to normal, the arrangement tended to be normal, and the number of Nissl-positive cells was also increased compared with that of SAMP8 mice. A β_{1-42} , a polypeptide containing 42 amino acids, is less abundant in AD patients than A β_{1-40} , but it has higher neurotoxicity. It can form extracellular aggregates by H-bonding and induce the deposition of amyloid fibrils and plaques. As a result, A β_{1-42} is also the main component of age spots^[4]. Unfortunately, *in vivo* experiments, the effects of HAS on A β deposition, hyperphosphorylation of Tau proteins in SAMP mice and their mechanisms had not been explored, which need to be followed up with in-depth investigations.

We used A β_{1-42} -induced mouse nerve cell HT22 cells to mimic nerve cell status in AD. Our experiments demonstrated that after A β_{1-42} stimulated HT22 cells, oxidative stress occurred in cells and apoptosis was induced, which in turn impaired cell viability. This result also confirmed that during the development of AD, A β can induce oxidative stress of neuronal cells and cause apoptosis. ROS is an important indicator and the predominant reason for oxidative stress, and there are some important antioxidative enzymes such as HO-1, CAT, SOD and GSH-Px can scavenge the excessive ROS in body^[35]. After HAS treatment, the ROS level in mitochondria and cells stimulated by A β_{1-42} were reduced, and the content of MDA (an end-product of the lipid peroxidation) in cells would also be reduced. Importantly, after incubation of HAS and HT22 cells, the activities of antioxidative enzymes such as CAT, SOD and GSH-Px in cells increased to varying degrees, jointly maintaining the redox balance in cells. There is a close link between oxidative stress and apoptosis, and apoptosis can be induced through the mitochondrial pathway, death receptor pathway, and endoplasmic reticulum pathway. When cells receive oxidative stress stimuli, oligomer complexes formed by Bax and Bak will aggregate into the outer mitochondrial membrane pores, while Bcl-2 regulates the excessive opening of mitochondrial permeability transition pores. Subsequently, the mitochondrial osmotic pressure changes and the MMP collapses,

which in turn promotes the transfer of cytochrome c from mitochondria into the cytoplasm. Immediately afterwards, apoptosis activators bind to cytochrome c to form apoptosis complex, which in turn triggers the Caspase cascade and ultimately induces apoptosis. In our experiments, HAS restored the MMP decline caused by A β_{1-42} , increased the Bcl-2/Bax ratio, and ultimately inhibited apoptosis in HT22 cells. However, it is undeniable that in our experiment, we have not yet elaborated the specific target of HAS, which needs to be explored in future experiments. Subsequently, the mechanistic level can be explored regarding HAS modulation of signaling pathways related to oxidative stress or apoptosis in HT22 cells and thus having a neuroprotective effect against AD.

In recent years, researchers have generally believed that the gut microbiota, as the second brain of humans, has a structural composition disorder that promotes the development of AD. After HAS intervention, we found that the richness and diversity of intestinal microbiota in SAMP8 mice were significantly improved. Previous studies have proved that compared with healthy individuals, the abundance of Bacteroidetes in AD patients is obviously decreased^[36], while the abundance of Firmicutes is obviously increased^[37]. Our results were consistent with this conclusion. Bacteroidetes and Firmicutes can induce the proliferation of Treg cells or the production of short-chain fatty acids, thereby exerting anti-inflammatory and immunomodulatory effects^[38-39]. After HAS treatment, Bacteroidetes abundance was increased in SAMP8 mice, while Firmicutes abundance decreased significantly. *Akkermansia* is a representative genus of the human gut microbiota, and its reduced content will induce inflammation and impaired glucose tolerance^[40]. At the same time, *Akkermansia* can also play multiple roles in AD. It can produce short-chain fatty acids, preserve colonic shielding permeability, and reverse cognitive deficits in AD mice^[41]. Surprisingly, HAS could increase the relative abundance of *Akkermansia* in SAMP8 mice, which also has an improving effect on learning and memory impairment in mice. In addition, the significant decreases in *Bacteroides*, *Alistipes*, and *Parabacteroides* were found in AD^[42-43], which is consistent with our results. With the intervention of HAS, the abundance of these microbiota was restored. It is worth noting that *Lactobacillus* is believed to have antioxidant effects^[44]. In our experiments, the relative abundance of *Lactobacillus* in SAMP8 mice increased significantly. The explanation for this phenomenon may be its response to immunomodulatory activity, and P301L Tau transgenic mice also showed an increase in *Lactobacillus*^[45]. The above data comprehensively showed that HAS could improve AD by restoring the balance of intestinal microbiota and increasing the proportion of beneficial microbiota.

There is growing evidence that the microbiota can bind microbes to their hosts through metabolites in the body. After analysis of plasma metabolites in mice, we found that HAS altered plasma metabolites in SAMP8 mice. The results showed that different metabolites in the HAS group and the Model group were more enriched in purine metabolism, including guanosine, inosine and hypoxanthine. Compared to the Model group, HAS significantly increased the content of 3-indoleacrylic acid, acetylcarnitine, ascorbic acid, L-ascorbate, lipoic acid and N-lactoyl-phenylalanine. The contents of guanosine, hypoxanthine, inosine, and PGA1 were decreased. In addition to 3-indoleacrylic acid and N-lactoyl-phenylalanine, the effects of other metabolites on AD and oxidative stress have been reported. Acetylcarnitine, for example, can prevent nerve cell

apoptosis by protecting mitochondrial function and alleviate the pathological phenotype associated with AD^[46-47]. Ascorbic acid and *L*-ascorbate are currently recognized antioxidants. They can play a neuroprotective role in AD by inhibiting oxidative stress, autophagy and apoptosis^[48-49]. Lipoic acid has been shown to improve AD cognition in clinical randomized controls. Studies on related mechanisms have shown that the protective effect of lipoic acid may be related to the regulation of synaptic transmission, lipid transporter activity, and neuroinflammation^[50]. Previous studies have shown that *N*-lactoyl-phenylalanine is strongly associated with metabolic markers that reflect mitochondrial overload, but this study mainly focused on acute myocardial infarction associated with diabetes^[51]. 3-Indoleacrylic acid, as a metabolite of tryptophan, can activate signaling pathway and control immune cell function through transcription factors such as aryl hydrocarbon receptor^[52]. However, the effects of 3-indoleacrylic acid and *N*-lactoyl-phenylalanine in AD have not been clearly reported, so we conducted a preliminary exploration of the activities of these 2 compounds. The results showed that 3-indoleacrylic acid could effectively inhibit the apoptosis of HT22 cells induced by A β , and then improve the viability of HT22 cells. In contrast, *N*-lactoyl-phenylalanine could inhibit apoptosis, but its effect was slighter than 3-indoleacrylic acid. Guanosine and inosine can inhibit the neuroinflammation in AD and improve learning and memory^[53-54]. Our experimental results showed that guanosine and inosine contents in SAMP8 mice were higher than others. However, the FC of guanosine and inosine are small, and the content is less than that of the other differentiated metabolites. Therefore, the anti-AD ability of SAMP8 mice was less affected. Furthermore, hypoxanthine is an important intermediate product in purine metabolism. Hypoxanthine can induce oxidative stress in rat striatum, enhance acetylcholinesterase activity in the hippocampus, and then aggravate AD^[55-56]. Opinions about PGA1 are controversial. PGA1 are substances released in the inflammatory response and are also seen as key factors in the early stages of neurodegenerative diseases. In earlier studies, PGA1 were thought to be neurotoxic, causing cytoplasmic vacuolation and neurite fragmentation^[57-58]. PGA1 can induce cancer cell death by triggering apoptosis^[59]. However, in recent experiments, PGA1 was found to promote cholesterol effluxion and reduce A β deposition, thus alleviating AD^[60]. These results suggest that HAS may protect neurons by influencing these metabolites to inhibit oxidative stress or apoptosis. However, what should not be ignored is how HAS plays a neuroprotective role in AD by affecting the microbial-gut-brain axis, hormones, brain metabolites and other related signaling pathways in mice, which needs to be further explored. To sum up, our research on the neuroprotective effects of HAS on AD is superficial. It is recommended to follow up with an in-depth exploration of the mechanism.

5. Conclusion

In conclusion, our present work suggested that HAS can inhibit apoptosis in nerve cells by attenuating A β induced oxidative stress level *via* influence the metabolic pathways and the gut microbiota composition of AD mice. Our results will help elucidate the pathway of HAS in the treatment of AD and provide a theoretical basis for further development of HAS.

Conflict of interest

The authors report no conflicts of interest in this work.

Acknowledgements

This work was supported by Natural Science Foundation of Sichuan Province (2022NSFSC0720), State Administration of Traditional Chinese Medicine of Sichuan Province of China (2020HJZX001) and Project of Sichuan Provincial Health Commission (21PJ130).

Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250214>.

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