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ORIGINAL RESEARCH

Multisensory Fusion Training and 7, 8-Dihydroxyflavone Improve Amyloid-β-Induced Cognitive Impairment, Anxiety, and Depression-Like Behavior in Mice Through Multiple Mechanisms

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Background: There is growing interest in the role of physical activity in patients with of Alzheimer's disease (AD), particularly regarding its impact of cognitive function, gut microbiota, metabolites, and neurotrophic factors.

Objective: To investigate the impact of multisensory fusion training (MSFT) combined with 7, 8-dihydroxyflavone (DHF) on the behavioral characteristics, protein expression, microbiome, and serum metabolome using the AD model in mice induced with amyloid-β (Aβ).

Methods: We assessed cognitive ability, anxiety-like and depression-like behaviors in Aβ mice using behavioral measures. Western blotting was employed to detect the expression of relevant proteins. The 16S rRNA gene sequencing and metabolomics were used to analyze changes in the intestinal microbial composition and serum metabolic profile, respectively, of Aβ mice.

Results: The behavioral outcomes indicated that a 4-week intervention combining DHF and MSFT yielded remarkable improvements in cognitive function and reduced anxiety and depression-like behaviors in Aβ mice. In the hippocampus of Aβ mice, the combined intervention increased the levels of BDNF, VGF, PSD-95, Nrf2, p-GSK3β and p-CREB proteins. Analyses of sequence and metabolomic data revealed that Bacteroides and Ruminococcaceae were remarkably more abundant following the combined intervention, influencing the expression of specific metabolites directly linked to the maintenance of neuronal and neurobehavioral functions. These metabolites play a crucial role in vital processes, such as amino acid metabolism, lipid metabolism, and neurotransmitter metabolism in mice.

Conclusion: Our study highlighted that MSFT combined with DHF improves cognitive impairment, anxiety, and depression-like behavior in Aβ mice through multiple mechanisms, and further validated the correlation between the gut microbiome and serum metabolome. These findings open up a promising avenue for future investigations into potential treatment strategies for AD.

Keywords: Alzheimer's disease, brain-derived neurotrophic factor, cognitive impairment, acousto-optic stimulation, treadmill exercise

Introduction

Alzheimer's disease (AD) is a leading neurodegenerative condition predominantly affecting older adults, leading to memory loss, cognitive decline, and behavioral challenges.[1–3] Currently, the number of individuals with AD worldwide is approximately 50 million, with projections indicating that this might triple by 2050.[4] Clinical manifestations of AD include declining memory, cognitive impairment, language difficulties, and reduced learning abilities.[5] AD is characterized by the formation of senile plaques, composed of extracellular amyloid-β (Aβ) oligomers, and the accumulation of hyperphosphorylated tau proteins, leading to the formation of intracellular neurofibrillary tangles.[6,7] The primary

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pharmacological therapies for AD involve the use of acetylcholinesterase (AChE) inhibitors, such as donepezil, rivastigmine, and galantamine, along with memantine, an antagonist targeting the excitatory amino acid receptor.[8] While drug therapy remains the mainstream approach for AD treatment, it only offers temporary relief of symptoms and cannot reverse the underlying pathological processes; furthermore, it often has significant side effects in the older population.[9] However, available therapeutic options for AD are limited. Studies have shown that adult neurogenesis can be enhanced through different approaches, such as the use of drugs, engaging in physical activity, and exposure to stimulating environments.[10,11] These interventions have demonstrated encouraging outcomes in improving cognitive abilities and decreasing Alzheimer's disease pathologies in animal models. However, the effectiveness of a singular treatment approach remains limited.[12]

Growing evidence suggests that exercise interventions can reduce Aβ deposition, enhance neural plasticity, stimulate neurogenesis, improve cognitive function, enhance daily life abilities, and reduce the incidence of neurobehavioral symptoms.[13,14] Studies have demonstrated that engaging in physical activity can lower the accumulation of Aβ and the excessive phosphorylation of tau proteins in the brain.[15,16] Exercise can also regulate the cholinergic system[17] and influence the expression of brain-derived neurotrophic factor (BDNF) and other factors derived from the brain.[18] After the exercise, the expressions of neuropeptides, such as VGF (non-acronymic), secretogranin II and NPY in the hippocampus, were significantly upregulated, with the expression characteristics of VGF resembling those of BDNF after exercise. Previous research involving mice with AD subjected to treadmill exercise showed improvement in cognitive and memory functions, along with enhanced hippocampal synaptic plasticity attributed to increased BDNF levels.[19] BDNF plays significant functions in the adaptive responses of neuronal circuits to physical activity, encompassing neurogenesis, the formation of synapses, cognitive processes and ability of neurons to withstand stress.[20,21] Brain tissue samples from individuals with AD show reduced levels of mature BDNF and the BDNF receptor tropomyosin-related kinase receptor type B (TrkB) compared with controls.[22] Despite evidence linking BDNF to exercise, the mechanism by which different-intensity training improves AD by regulating BDNF remains unknown. However, depending on the severity of dementia, the outcomes of exercise interventions can vary by the type of exercise, level, regularity, etc. For instance, an 8-week regimen of strength training was found to reduce AChE enzyme activity, whereas in a model of Aβ-mediated rats with AD, engaging in an equal amount of running exercise demonstrated greater efficacy in improving antioxidant capacity. These findings suggest that various forms of exercise elicit diverse neuroprotective outcomes.[23]

Acousto-optic stimulation has emerged as a potential method for safeguarding against AD. The disruption of gamma rhythms in the brain affected by AD, caused by the buildup of Aβ, hinders neural activity associated with memory formation via gamma oscillation.[24] Recently, studies have pointed out that 40 Hz sensory stimulation (such as sound and visible light) can improve the abnormal brain gamma rhythms in patients with AD, thus improving their clinical symptoms.[25] Particularly, Dr. Li-Huei Tsai and her team discovered that applying 40 Hz acousto-optic stimulation could trigger gamma brainwave frequencies in mice with AD, lower Aβ protein levels in the brain, and enhance the performance of microglial cells. Furthermore, it could reduce the accumulation of Aβ and tau proteins, and this stimulation not only effectively enhanced the functionality of neurons and synapses but also significantly promoted the "immune phagocytic" function of microglial cells, thereby effectively improving the cognitive abilities of the mice. However, another research team found that 40 Hz light stimulation neither reduced levels of Aβ 40/42 deposition in the brain tissue of mice with AD, nor did it change the morphology of microglia or impact gamma waves in the neocortex and hippocampus.[26–28] Despite these contrasting findings, the consistent positive effects of acousto-optic stimulation in humans have yet to be fully confirmed.

Research has indicated that gut dysbiosis, an imbalance in the gut microbiota, can promote the release of lipopolysaccharides and amyloids, leading to increased permeability of both the gut and the blood–brain barrier.[29,30] Exercise has been demonstrated to improve the clinical symptoms of AD by stimulating a variety of gut bacteria, regulating the composition of the microflora, and affecting metabolites. Moreover, it has been suggested that the gut microbiome influences the pathophysiology of AD by affecting organ metabolism. The modulation of this process can occur by interacting with the intestinal barrier or by releasing bioactive substances, such as BDNF, into the circulatory system.[31–33] However, the relationship between acousto-optic stimulation and the intestinal flora remains unclear. Furthermore, the unexplored territory lies in the molecular, microbiome, and metabolic mechanisms triggered by a multifactor intervention involving treadmill exercise of varying intensities, along with acousto-optic stimulation, in the presence of toxic Aβ oligomers.

Multisensory fusion training (MSFT) is a novel intervention method based on different intensity treadmill exercises and specific frequency audio-visual stimuli. It was developed in the laboratory through previous exploration[34] and experience summary.[10] This intervention combines exercise and multimodal interventions with 40 Hz audio-visual stimulation, aiming to overcome the singularity, excessive exercise intensity, or long intervention period of traditional exercise methods, making it more suitable for elderly people's physiological characteristics and improving intervention effectiveness. Previous studies by our research group have shown that multisensory fusion training can promote neurogenesis in AD model mice, increase the expression of hippocampal-related neuronal proteins, neurotrophic factors, and anti-apoptotic proteins, thereby improving cognitive function impairment, and reducing anxiety and depression-like behavior.[10] Literature reports suggest that treatment that increases BDNF levels may help improve clinical outcomes in AD patients. However, the delivery of exogenous BDNF is limited due to its short half-life in plasma and restricted diffusion across the blood-brain barrier. Therefore, many intervention strategies aim to restore endogenous BDNF levels and signal transduction. These treatments target BDNF by promoting endogenous BDNF production directly (such as BDNF gene therapy) or indirectly enhancing BDNF signal transduction and secretion in the brain.[35] 7.8-Dihydroxyflavone (DHF) is a natural flavonoid compound that is a potent neurotrophic factor, easily crossing the blood-brain barrier with important biological functions in the nervous system. It is often used as a TrkB receptor agonist to mimic BDNF expression, and its oral efficacy has been confirmed in animal experiments.[36,37] MSFT and DHF intervene in AD from different perspectives, and their combined use can comprehensively utilize their advantages and enhance treatment effectiveness. MSFT can promote neurogenesis by stimulating neural network connections, while DHF can provide neuroprotection and proliferation.[37] The combination of the two can mutually promote the process of neurogenesis and reduce inflammation and oxidative stress levels. Therefore, this study comprehensively analyzed the effect of DHF combined with MSFT on improving cognitive function in Aβ-induced mice and further validated the correlation between the gut microbiome and serum metabolome, thus opening up a promising avenue for future research into potential therapeutic strategies for AD.

Materials and Methods

Animals

ICR male mice with a weight ranging from 22 to 28 g, were bred at Ningbo University's Experimental Animal Center. The breeding environment was maintained at a temperature range of (23 ± 2) °C, with a humidity lever of (55 ± 15) %, and a 12-hour light/dark cycle. Before commencing the experimental procedures, the mice were given unrestricted access to food and water and were allowed to adapt to the surroundings for a period of one week. In accordance with the National Institutes of Health's regulations for the welfare and utilization of lab Animals (NIH Publications No. 80–23), animal tests are conducted.

Reagents

The $A\beta_{1\text{-}42}$ monomer was obtained from GL Biochem (Shanghai) Ltd. and dissolved in hexafluoroisopropanol (HFIP, Sigma-Aldrich) to create a solution with a concentration of 50 μM. The solution was allowed to sit at room temperature for 20 minutes. Subsequently, 100 microliters of the solution were transferred to an EP tube, followed by the addition of 900 microliters of sterile deionized water. After standing at room temperature for an additional 20 minutes, the tube was placed on a nitrogen evaporator and evaporated for 20 minutes. The solution in the tube was centrifuged at a force of 14,000 G for 15 minutes, and the liquid above was transferred to another EP tube. For later use, the prepared solution was incubated on a magnetic shaker at 500 rpm for 48 hours.[38]

7.8-Dihydroxyflavone (DHF, Sigma-Aldrich) is a natural flavonoid compound that acts as a TrkB agonist. To prepare the DHF solution, 1% DMSO (Sigma-Aldrich) was added to a saline solution and stored at 4°C. DHF was administered orally at a dose of 5 mg/kg in a volume of 5 mL/kg/day at a consistent time each day. The control groups received an equivalent volume of blank solvent.

Multisensory Fusion Training (MSFT) Protocol

The Multisensory fusion training (MSFT) protocol was performed as described previously with some modifications.[10] MSFT protocol incorporated various levels of treadmill exercise and stimulation through auditory and visual flickering at a frequency of 40 Hz. Running on the treadmill was performed according to the previous protocol.[39,40] To demonstrate their adaptability to running, the animals were accustomed to using a rodent motorized treadmill. After a 3 meter per minute speed for 5 minutes, there was a 5 meter per minute speed for another 5 minutes on the initial day. During the training, animals ran on treadmills for 30 minutes/day, 6 days per week at different intensities. For 5 minutes, they ran at 10 meters per minute, followed by a 4-minute rest. Afterwards, the mice were subjected to a velocity of 15 meters per minute for a duration of 5 minutes, which was succeeded by a 4-minute period of rest. This sequence was repeated a total of three times. After a 1-hour rest, the mice were moved to experience flickering stimulation of both auditory and visual senses. We used a previous study's method for presenting flickering stimulation in both auditory and visual formats at a frequency of 40 Hz.[26] Light-emitting diode (LED) bulbs were used to produce a 40 Hz visual flicker in a calm and softly illuminated room. This flicker followed a pattern of 12.5 ms of light, followed by 12.5 ms of darkness, and had an intensity of 60 W. Simultaneously, the mice experienced a 40 Hz auditory tone train, delivered through a speaker (AYL, AC-48073), with each tone lasting for 1 ms and presented at a volume of 40 dB. The 40 Hz light flicker and auditory tones lasted 1 hour a day, 6 days a week, until they were sacrificed. Other experimental mice were both subjected to randomized light flicker and tone stimulations while being placed in a dark chamber. The bursts of light and sound were administered at irregular intervals, chosen from a consistent range with an average interval of 25 milliseconds. The duration of each pulse was 25 ms, and the sound intensity varied at different levels. The same as the experimental group, control groups were given 1 hour of stimulation daily, 6 days a week until they were sacrificed.

Surgery and Experimental Design

Male mice in the ICR group were randomly divided into five groups: saline control without any intervention (saline), Aβ injection without any intervention (Aβ), Aβ injection with DHF treatment (Aβ + DHF), Aβ injection with MSFT treatment (Aβ + MSFT), and Aβ injection with combined DHF and MSFT treatment (Aβ + DHF + MSFT). The AD model was established by injecting 1 μL/side of Aβ-oligomer and control solvent into the CA1 region of the hippocampus (coordinates: AP −1.5 mm, ML ±1.2 mm, DV −1.5 mm). After a recovery period of 7 days, the mice received long-term treatment with DHF (5 mg/kg, oral administration) and MSFT. Behavioral tests were conducted after a 4-week duration. MSFT involved various levels of treadmill exercise (10 meters per minute for 5 minutes, 15 meters per minute for 5 minutes, three times per day, six days per week) along with 40 Hz acousto-optic stimulation (one hour per day, six days per week). On day 29, behavioral tests including OF (open field test), NOR (novel object recognition test), YM (Y-maze test), EPM (elevated plus-maze test), TST (tail suspension test), FST (forced swim test), and MWM (Morris water maze test) were conducted. Two observers, blinded to the treatment conditions, performed all behavioral studies. Statistical analysis was conducted using pooled data. After completing the behavioral assessments, the mice were euthanized, and specimens were collected for further investigations. Biochemical analysis was performed on hippocampal tissues, 16S rRNA gene sequencing was conducted on feces samples, and metabolomic sequencing was performed on serum samples (the experimental procedure is illustrated in Figure 1).

Behavioral Tests

Open-Field (OF) Test

The OF test followed established protocols,[41] where mice were placed in a 60×60×15 cm3 arena with a yellow dim light. The floor of the arena was divided into four equally sized squares. Line crossings and rears were documented within 5 minutes. The apparatus was cleaned with a 10% ethanol solution after each mouse test.

Novel Object Recognition (NOR) Test

Modifications were made to the NOR test,[42] which consisted of three consecutive days: familiarization, instruction, and memory sessions. Prior to the experiment, mice underwent an OF test to establish their baseline exploratory behavior. After a 24-hour interval, each mouse was placed in an OF arena with two identical objects in opposite corners for 5

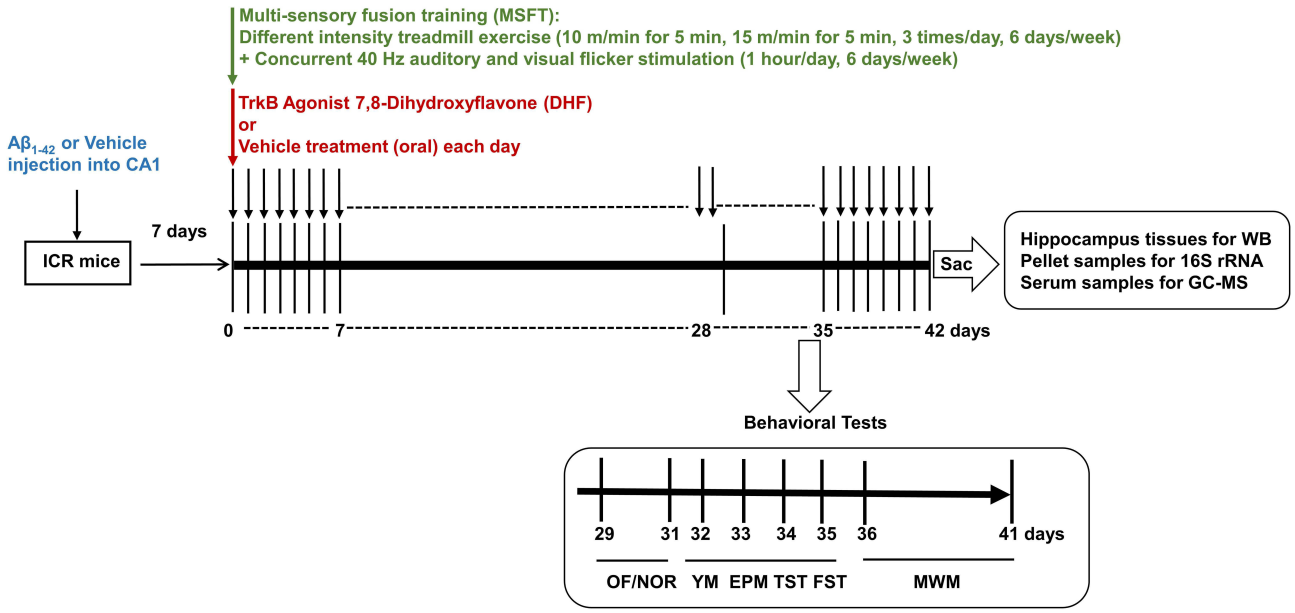

Figure 1 Experimental procedures and behavioral protocol. Aβ oligomers were microinjected into the CA1 region of the mouse hippocampus, after 7 days the mice were chronically treated with 7.8-Dihydroxyflavone (DHF, 5 mg/kg, i.g.), or vehicle every day. A multisensory fusion training (MSFT) protocol was also carried out, involving treadmill exercises at two different speeds (10 m/min and 15 m/min for 10 min, 3 times per day, 6 days per week), and 40 Hz acousto-optic stimulation (1 hour per day, 6 days per week). Day 29 began behavioral tests, including. Following the Conclusion of behavioral assessments, samples were collected from euthanized mice for further studies (Sac).

Abbreviations: OF, open field test, NOR, novel object recognition test, YM, Y-maze test, EPM, elevated plus-maze test, TST, tail suspension test, FST, forced swim test, and MWM, Morris water maze test.

minutes of unrestricted exploration. On the third day, one of the objects was replaced with a novel object (NO) that differed in color and shape. The mice were given another 5 minutes to explore the arena and interact with the objects. To minimize olfactory cues, the arena and objects were cleaned with a 10% ethanol solution after each trial.

Y-Maze (YM) Test

The YM experiment followed previous instructions with some modifications. The Y-maze consisted of three arms, each with a 120° angle between them. The dimensions of each arm were 60 cm (length) x 10 cm (width) x 35 cm (height). The experiment consisted of two phases with a 1-hour interval (1 h ITI) in between. During the training phase, a new limb was blocked by a divider. The rodent was placed in the starting limb and allowed to freely explore for 6 minutes, except for the blocked limb. After the training period, the mouse was returned to its original housing. After a 1-hour interval, the testing phase began, where the partition blocking the novel arm was removed. The rodent was placed in the starting limb and allowed to freely investigate all three limbs for 3 minutes. Video recordings were used to measure the time spent by each mouse in each arm and the number of transitions between arms within the 3-minute period.

Elevated Plus-Maze (EPM) Test

The EPM was used to assess anxiety-like behavior in mice.[43] Mice were tested in a plus-shaped device made of dark-gray PVC material positioned 70 cm above the ground. The maze had two enclosed limbs, each measuring 50 cm x 10 cm, and two open arms without walls (closed limbs). A central open area measuring 10 cm x 10 cm separated the open and closed arms. At the start of the test, the mice were placed facing an open arm in the center of the maze. Their behavior was observed, tracked, and recorded for 5 minutes. The duration spent on each arm and the count of entries were documented. An entry was counted when all four paws of the mouse fully entered a particular arm. The analysis focused on two primary indices of trait anxiety-like behavior in rodents: the ratio between the time spent in open arms and the total time spent in both arms (Percentage of Open-arms time %) and the ratio between the entries made into the open arms and the total number of entries in both arms (Percentage of Open-arms entries %).

Tail-Suspension Test (TST)

The TST experiment was conducted with slight modifications following a previously described protocol.[44] Mouse tails were positioned approximately 1 cm away from sticky tape and suspended 50 cm above the ground. Each mouse was allowed to hang for a total duration of 6 minutes, and the period of immobility was observed during the last 4 minutes of the test. Immobility was defined as the absence of any movement in the limbs or body. An observer, who was blinded to the treatment conditions, scored the test using a video camera.

Forced-Swim Test (FST)

The FST examination followed a previously described method.[45] Each mouse was placed individually in a transparent plastic tube. After a 6-minute swimming session, the duration of immobility during the last 4 minutes was measured. Immobility was defined as the state when the mouse stopped struggling and remained motionless, only moving its head above the water when necessary. Fresh water was used for each mouse to eliminate potential olfactory cues. The testing order was randomized to minimize any impact, and the observer evaluating the results was blinded to the treatment conditions to ensure unbiased assessment.

Morris Water-Maze (MWM) Test

The MWM test was conducted with minor modifications following a previously described protocol.[46] Initially, the mice underwent a training phase to learn the location of a visible platform that was randomly placed in a specially designed pool for mice. Subsequently, the mice received training to locate a submerged platform within one of the four sections of the pool over four consecutive days. To find the submerged platform, each mouse completed four trials per day for four training days. If a mouse failed to locate the platform within the given time, it was gently guided towards the platform and allowed to stay there for 20 seconds. A probe trial was conducted 24 hours after the final hidden platform trial to assess spatial memory. During this trial, the platform was removed, and the mice were given 90 seconds to explore the pool. A heating lamp was used to maintain a warm temperature for the mice during testing, and all testing sessions were conducted at the same time each day. Throughout the entire testing process, various parameters, including time and path spent in different zones and quadrants, swimming paths, latencies, and entries into the target quadrant, were meticulously recorded.

Western Blot

The procedure was carried out as previously explained with certain alterations.[47] We sonicated hippocampal brain tissues with protease and phosphatase inhibitors in RIPA lysis buffer (Beyotime Biotech, Jiangsu, China). After being subjected to centrifugation at a force of 16,000 g for a duration of 30 minutes, the samples underwent separation of the total supernatant protein using SDS-PAGE. A PVDF membrane (Millipore, CA) was used to transfer the separated protein. Subsequently, the PVDF membranes were incubated overnight at a temperature of 4°C with the following antibodies: rabbit anti-BDNF antibody (1:1000, Abcam, ab108319), rabbit anti-VGF antibody (1:1000, Abcam, ab74140), rabbit anti-PSD-95 antibody (1:1000, Cell Signaling Technology, 3450), rabbit anti-Nrf2 antibody (1:1000, Abcam, ab62352), rabbit anti-p-Ser9-GSK3β antibody (1:1000, Cell Signaling Technology, 5558), rabbit anti-GSK3α/β antibody (1:1000, Cell Signaling Technology, 5676), mouse anti-phospho-CREB (Ser133) antibody (1:1000, Cell Signaling Technology, 9196), and anti-beta actin (1:5000, Invitrogen, MA5-15,739). A 60-minute incubation at ambient temperature was followed by the addition of IRDye 680RD goat anti-rabbit IgG (1:15,000, LI-COR Biotechnology, Lincoln, NE) or IRDye 800CW goat anti-mouse IgG (1:15,000, LI-COR Biotechnology, Lincoln, NE) to the membranes. Identification and measurement of the distinct bands were conducted using a fluorescence scanner (Odyssey Infrared Imaging System, LI-COR Biotechnology, Lincoln, NE). Each of the samples was analyzed at a minimum of three times.

Fecal Microbiome Analysis

At the end of the experiment, fecal samples were collected and genomic DNA was extracted using the CTAB method. PCR amplification was performed with high-fidelity DNA polymerase and specific primers containing barcodes targeting

the V3-V4 variable region of the sequencing region. The PCR products were separated on 2% agarose gels, and the targeted fragments were extracted using the AxyPrepDNA Gel Recovery Kit. The PCR products were quantified using the QuantiFluor™ -ST Blue Fluorescence Quantification System and combined in required ratios for sequencing. Libraries were constructed using the NEB Next® UltraTM DNA Library Prep Kit and assessed for quality using Qubit and Agilent Bioanalyzer 2100. Microbiome diversity was analyzed using QIIME 2, Mothur, and Bray–Curtis metrics to evaluate alpha and beta diversity, including the OTU table to assess variations in microbial genera. Statistical significance was determined using the Adonis function.

Serum Metabolomics Analysis

After harvesting blood from mouse eyeballs, let it stand at room temperature for 30 minutes until it coagulates. Centrifuge the coagulated blood at 3000 RPM for 10 minutes, collect the supernatant, and add it to a pre-cooled mixture of methanol, acetonitrile, and water (2:2:1, v/v). Vortex and sonicate the mixture at low temperature for 30 minutes, then allow it to sit at −20°C for 10 minutes. Centrifuge the mixture at 14,000 g and 4°C for 20 minutes, collect the supernatant, and vacuum dry it. For mass spectrometry analysis, reconstitute the dried sample by adding 100 μL of acetonitrile-water solution (acetonitrile:water = 1:1, v/v). Vortex it, centrifuge at 14,000 g and 4°C for 15 minutes, and collect the supernatant for injection analysis. The samples were separated using an Agilent 1290 Infinity LC Ultra-High Performance Liquid Chromatography (UHPLC) system with a HILIC column, and the sample-level and MS/MS spectra were collected using an AB Triple TOF 6600 mass spectrometer. The data extracted by XCMS were first subjected to metabolite structure identification and data preprocessing. This was followed by experimental data quality evaluation and finally, data analysis was performed.

Statistical Analysis

Mean ± standard error of the means (SEM) was used to express all data, which were then used GraphPad Prism to analyze the data using a one-way analysis of variance (ANOVA) and Dunnett's test and Tukey's test. Post hoc multiple treatment comparisons were conducted using data obtained from the MWM acquisition training. Statistically significant $P<0.05$ was considered.

Results

Combined Treatment of DHF and MSFT Mitigates the Cognitive Deficits Induced by Aβ Oligomers in Mice, Enhancing Learning and Memory Abilities

To evaluate the potential of MSFT and DHF treatment in improving cognitive decline induced by Aβ oligomers, post-intervention mice underwent the NOR, YM, and MWM tests. Figure 2A outlines the experimental timeline for conducting the animal behavior experiments. After 1 week of administering Aβ oligomers, the mice received treatment with MSFT or DHF for 4 weeks. Subsequently, they underwent NOR, YM, and MWM tests. The NOR test revealed no significant variances in the number of line crossings and rearing behaviors between the groups, suggesting that the MSFT and DHF treatments had no impact on the spontaneous activity of the mice (Figure 2B and C, $P > 0.05$). During the "training phase" with two identical objects, the recognition index showed no significant differences between the groups (Figure 2D, $P > 0.05$). Nevertheless, the preliminary session unveiled that mice induced by Aβ oligomers exhibited a notably reduced recognition index than those injected with saline (Figure 2E, $P < 0.05$). Furthermore, mice induced with Aβ oligomers and treated with DHF or DHF combined MSFT exhibited an higher recognition index than those induced with Aβ oligomers without any intervention (Figure 2E, $P < 0.001$, $P < 0.01$), implying that the combination of DHF and MSFT mitigated the cognitive deficits induced by Aβ oligomers in mice, enhancing learning and memory abilities.

Furthermore, we conducted a YM test to assess the impact of MSFT or DHF on the cognitive function of Aβ-induced mice. During the testing period, the duration and entries of the targeted arm in Aβ-induced mice were significantly lower than those in the saline-injected mice (Figure 2F, $P < 0.05$, Figure 2G, $P < 0.01$), indicating that cognitive impairments in mice were induced by Aβ oligomer alone. In contrast, Aβ-induced mice that received both MSFT and DHF showed

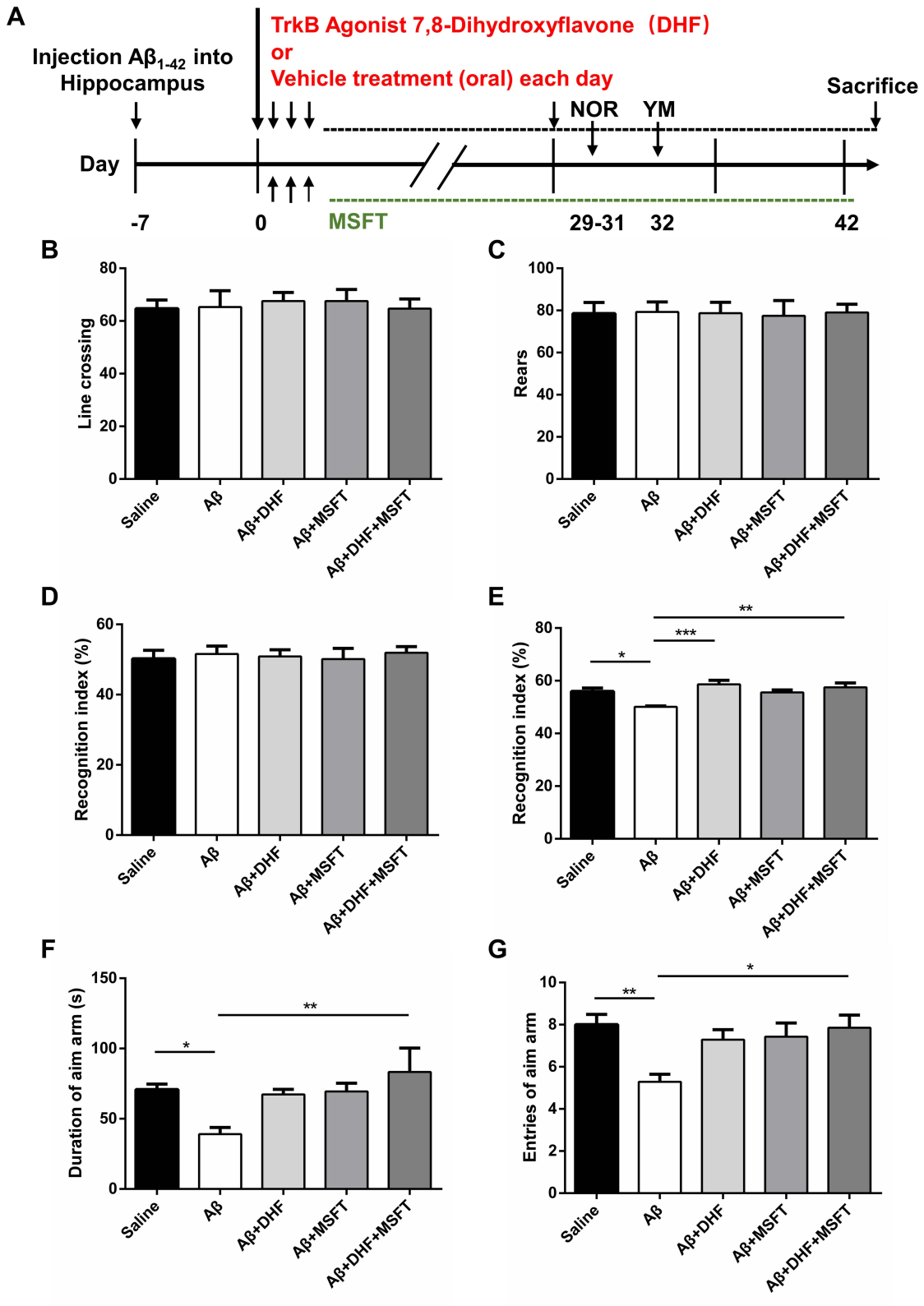

Figure 2 Improvement in learning and working memory in Aβ-induced mice by combining DHF and MSFT interventions. (**A**) Experimental schedule for the NOR and YM programs. **(B** and **C)** Number of line crossings and rear behaviors during the OF test on the first day. (**D**) Duration of exploration of two identical items during the NOR test on the following day. (**E**) Exploration time for two distinct items measured during the NOR test on the third day. (**F**) Time spent by each group of mice in the target arm during the YM test. (**G**) The number of entries from each group of mice in the target arm during the YM test. *P < 0.05, **P < 0.01, ***P < 0.001.

a prolonged duration and higher number of entries in the targeted arm compared with Aβ-induced mice subjected to alternative interventions (Figure 2F, $P < 0.01$, Figure 2G, $P < 0.05$). This suggests that the combination of MSFT and DHF has the potential to alleviate Aβ-induced working memory impairment in mice.

Furthermore, we used the MWM test to investigate the effects of MSFT or DHF on Aβ-induced spatial learning and memory in mice. Figure 3A outlines the experimental timeline for conducting the animal behavior experiments. Figure 3B presents illustrative images depicting the swimming paths of each experimental group during the spatial probe trial on the fifth day. Remarkably, during the training session, the different groups of mice demonstrated a comparable duration in initially reaching the platform (Figure 3C). The swim velocity during the training session showed no significant variations among the groups, indicating that there was no discernible difference in motor ability among them (Figure 3D, $P > 0.05$). Furthermore, during the training session on the fourth day, escape latency was assessed, revealing a noteworthy difference: mice in the Aβ group reached the platform significantly later than did the saline-treated group (Figure 3E, $P < 0.05$). In contrast, a significant reduction in latency to locate the target platform was observed for Aβ-induced mice treated with MSFT or DHF and MSFT combined, compared with that of Aβ-induced mice (Figure 3E, $P < 0.05$, $P < 0.05$). During the exploration period, we found that Aβ-induced mice with DHF and MSFT intervention had significantly longer durations and more entries in the target quadrant than Aβ-induced mice without any form of intervention (Figure 3F, $P < 0.01$, and Figure 3G, $P < 0.05$).

Implementation of DHF and MSFT Intervention Mitigates Anxiety and Depression-like Behaviors in Aβ-Induced Mice

To evaluate the efficacy of DHF and MSFT therapy in decreasing anxiety-like and depression-like behaviors in Aβ-induced mice, we conducted assessments utilizing the EPM, TST, and FST. Figure 4A illustrates the experimental timeline for conducting animal behavior experiments. EPM analysis of the percentage of time spent in the open arms and the number of entries into the open arms revealed a significant decrease in both aspects in the mice induced by Aβ oligomers compared to those treated with saline (Figure 4B and C, $P < 0.05$). However, MSFT intervention, with or without DHF, successfully reversed these alterations, resulting in a significant increase in the number of entries (Figure 4C, $P < 0.05$, $P < 0.05$) and time spent in the open arms (Figure 4B, $P < 0.05$, $P < 0.01$). Collectively, our findings validate that Aβ oligomers triggered anxiety-related conduct in mice, and MSFT intervention ameliorated these symptoms.

When examining behaviors resembling depression in TST and FST, it was observed that the duration of immobility in the Aβ-induced group of mice was significantly longer than that of the saline group (Figure 4D, $P < 0.01$ and Figure 4E, $P < 0.05$), indicating that Aβ oligomers caused depression-like phenotypes in mice. We found that during both the TST and FST, the implementation of interventions such as DHF, MSFT, and the combined application of DHF and MSFT stimulation resulted in a significant reduction in immobility time, compared with no intervention, in Aβ-induced mice (Figures 4D and E, $P < 0.01$, $P < 0.001$). This observation strongly suggests that the aforementioned treatments play a contributory role in exerting an antidepressant effect on Aβ oligomer-induced mice. Furthermore, it is worth noting that the implementation of the combined DHF and MSFT intervention exhibited superior efficacy in terms of their antidepressant (Figures 4D and E) and anxiolytic effects (Figures 4B and C), surpassing those of single-factor interventions. Collectively, our data unequivocally demonstrate that combined DHF and MSFT intervention significantly attenuates anxiety and depression-like behaviors in Aβ-induced mice.

Implementation of DHF and MSFT Intervention Increases the Levels of BDNF, VGF, PSD-95, Nrf2, p-Ser9-GSK3β and p-CREB Protein Levels in Aβ-Induced Mice

To validate the impact of DHF and MSFT intervention on Aβ-induced hippocampal protein expression in mice, we quantitatively assessed BDNF, VGF, PSD-95, Nrf2, p-Ser9-GSK3β and p-CREB protein levels by Western blot analysis. BDNF is a neurotrophic factor that plays a critical role in the development and ongoing functioning of neurons[48] and it does so by activating its receptor, TrkB. This activation is crucial for mediating neuroprotective functions and facilitating neuronal maturation and maintenance.[49] The neuropeptide VGF promotes neuronal growth and connectivity, PSD-95 (post-synaptic proteins) is vital for synaptic plasticity,[50] and Nrf2 is an important transcription factor that regulates

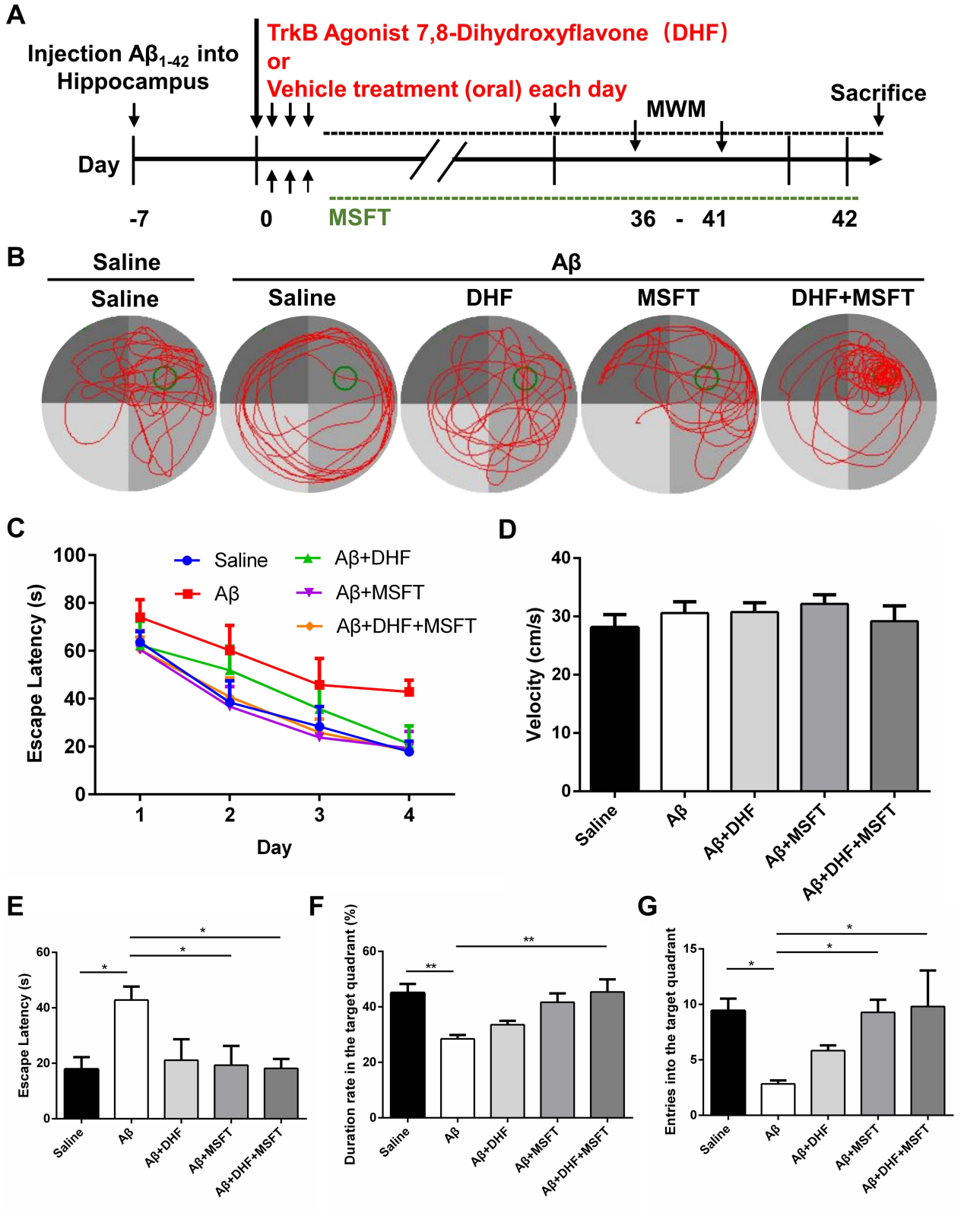

Figure 3 Improvement in cognitive impairment of spatial learning and memory in Aβ-induced mice after administering DHF and MSFT intervention. (**A**) Experimental schedule for the MWM program. (**B**) Trajectory of the MWM test for each group on the fifth day. (**C**) Time taken by the mice in each group to find the target platform during the training phase. (**D**) Swim velocity during the training phase. (**E**) Time spent finding the platform at the end of the training phase. (**F**) Traveling distance in the target quadrant during exploration. (**G**) Number of mice crossing the platform in the target quadrant. *P < 0.05, **P < 0.01.

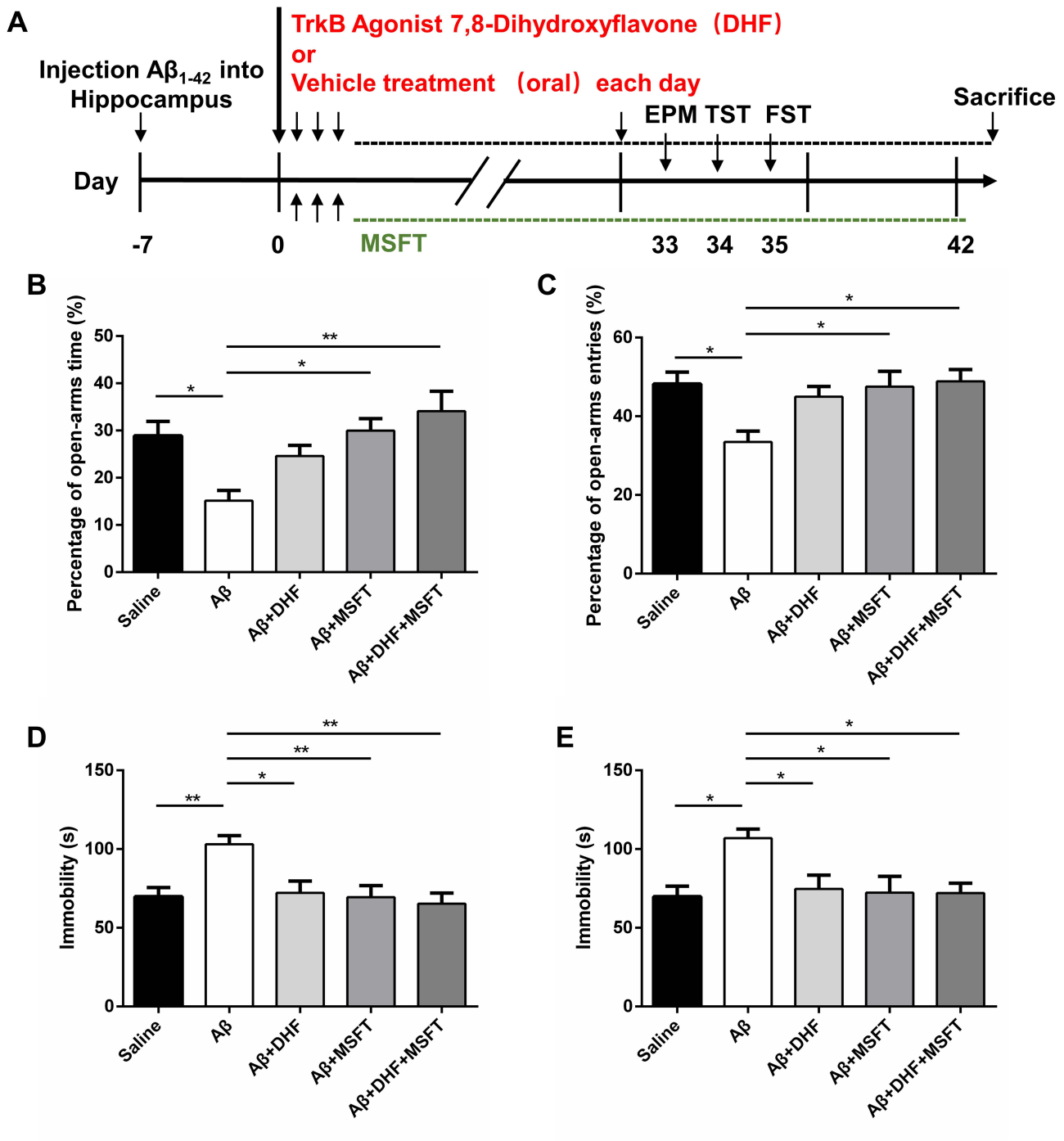

Figure 4 Amelioration of anxiety and depression-like behavior in Aβ-induced mice by combining DHF and MSFT intervention. (**A**) Experimental schedule. (**B**) Percentage of time spent by each group of mice in the open arms during the EPM. (**C**) Percentage of entries into the open arms during the EPM. Immobility time in the TST (**D**) and FST (**E**). *P < 0.05, **P < 0.01.

cellular oxidative stress response. The activation status of GSK3β is closely related to neurodevelopment, and p-CREB activity is closely associated with long-term potentiation (LTP) and synaptic plasticity. Figure 5A shows representative images of the protein expression of BDNF, VGF, PSD-95, Nrf2, p-Ser9-GSK3β, GSK3α/β, p-CREB and CREB as manifested by the bands obtained by immunoblotting. The Results showed increased expression of BDNF (Figure 5B, P < 0.01) and VGF (Figure 5C, P < 0.01) in the Aβ-induced mice treated with DHF and MSFT compared with the Aβ group, indicating that DHF and MSFT enhanced the levels of BDNF and VGF protein expressions in the Aβ-induced

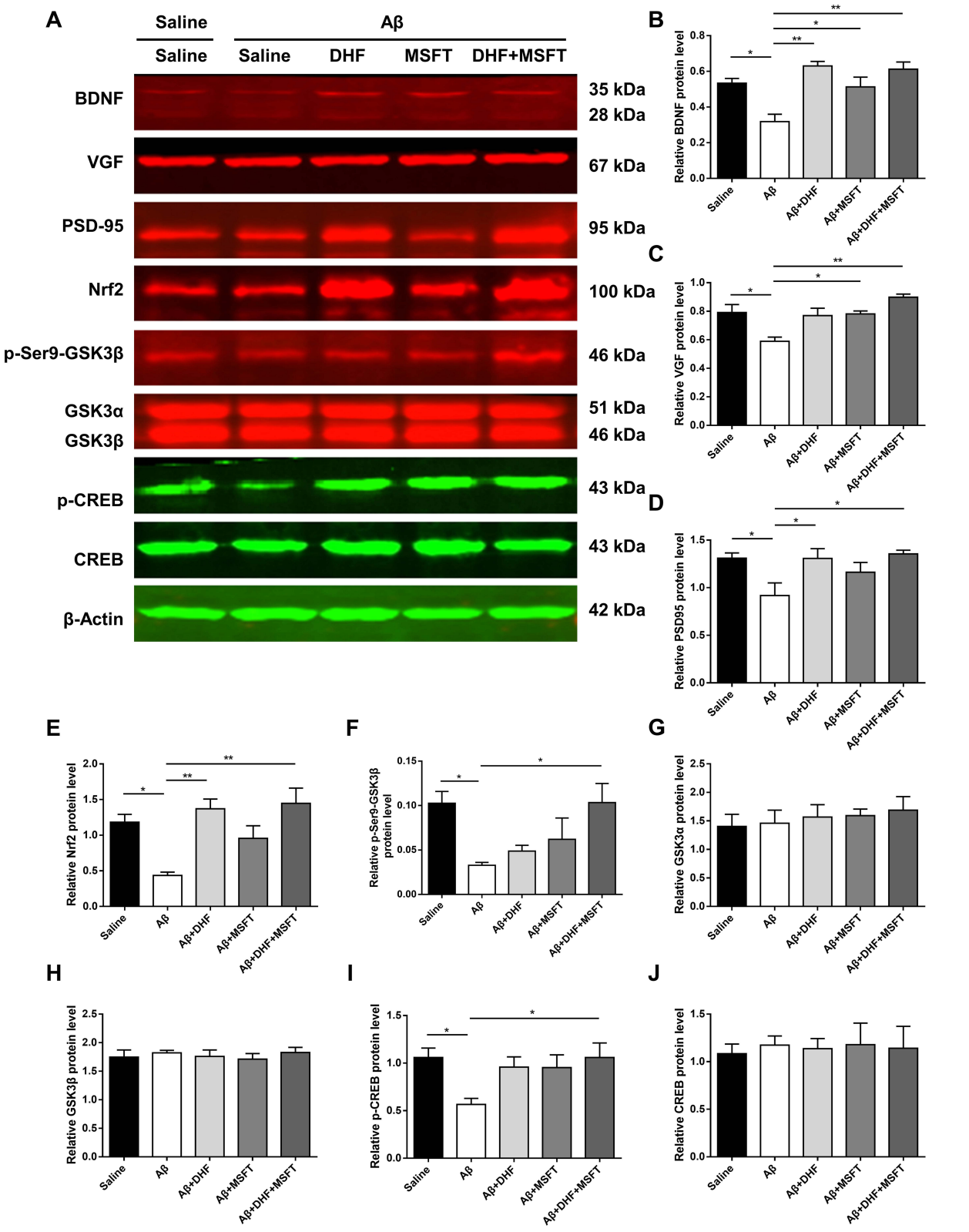

Figure 5 Increase in BDNF, PSD95, VGF, and p-CREB protein levels in the hippocampus of Aβ-induced mice due to the combined application of DHF and MSFT intervention. (**A**) Western blot expression images of the respective proteins in mouse hippocampal tissue. (**B–J**) Expression levels of BDNF (28 kDa), VGF, PSD-95, Nrf2, p-Ser9-GSK3β, total GSK3α/β, p-CREB and CREB protein in the mouse hippocampal tissue. *P < 0.05, **P < 0.01.

mice. Furthermore, the DHF alone and combined DHF and MSFT treatment significantly increased the levels of PSD-95 (Figure 5D, $P < 0.05$, $P < 0.05$) and Nrf2 (Figure 5E, $P < 0.01$, $P < 0.01$) compared with the mice induced by Aβ without any intervention. The combined DHF and MSFT treatment significantly increased the levels of p-Ser9-GSK3β (Figure 5F, $P < 0.05$) and p-CREB (Figure 5I, $P < 0.05$) compared with the mice induced by Aβ without any intervention. However, there was no significant difference between total GSK3α/β and CREB protein expression (Figure 5G, H and J, $P > 0.05$). These findings suggest that the joint DHF and MSFT treatment has the potential to enhance the expression of proteins associated with synaptic function, neuropeptides, and cellular transcription in the presence of Aβ toxicity.

DHF and MSFT Intervention Leads to Changes in the Abundance of Intestinal Flora in Aβ-Induced Mice

We assessed how DHF and MSFT influenced the proportion of the mouse gut microbiota at the phylum and family levels. To examine the potential effects of DHF and MSFT on the gut microbiota of mice, we evaluated the phylum and family levels to understand their impact on the composition of mouse gut microbiota. We selected the top 10 species at the phylum level and the top 20 species at the family level (with the remaining species categorized as "other") for bar chart visualization. As depicted in Figure 6A, the gut microbiota predominantly comprised taxa belonging to the phyla Bacteroidetes, Firmicutes, Verrucomicrobia, Epsilonbacteraeota, Deferribacteres, Patescibacteria, Deltaproteobacteria, Actinobacteria, and Gammaproteobacteria. The majority of sequences in the fecal microbiota belonged to the phyla Firmicutes, Bacteroidetes, and Proteobacteria, underscoring their dominance as key constituents of the gut microbial community. At the Family level (Figure 6B), compared with the saline-treated group (0.0250), the Aβ-induced group (0.0030) exhibited a nearly 8-fold decrease in relative abundance levels of Akkermansiaceae, indicating an imbalance in the gut microbiota. However, following the combined intervention with DHF and MSFT, the abundance of Akkermansiaceae reverted to approximately 4–10 times of its initial level. The abundance of Bacteroidaceae after DHF and MSFT combined intervention (0.0269) was over three times higher than that in Aβ oligomer-induced group (0.0081). Furthermore, compared with the saline-treated group (0.00061), in the Aβ oligomer-induced group (0.00018), the relative abundance levels of Deferribacteraceae within the phylum Deferribacteres underwent an approximately 4-fold decrease. However, following the combined intervention with DHF and MSFT, the abundance of Deferribacteraceae showed a substantial increase (0.0059), reaching approximately 33 times its initial level. Conversely, Staphylococcus, as a Gram-positive sphere, exhibited an approximately three times lower abundance of the Staphylococcus family after the combined intervention of DHF and MSFT (0.0014) compared with that in the Aβ oligomer-induced group (0.0036). The relative abundances of Rikenellaceae and Muribaculaceae showed minimal variation among the groups.

To explore the impact of DHF and MSFT on the relative abundance of the mouse gut microbiota at the genus level, a clustering analysis was conducted using species annotation and abundance information at the genus level, selecting the top 30 genera based on their abundance rankings. The results were visually represented through a heat map, as shown in Figure 6C. Compared with the Aβ-induced group, the Aβ plus DHF and MSFT group exhibited higher abundance of Bacteroides, Butyricicoccus, Ruminococcaceae, Akkermansia, Prevotellaceae UCG-001, Roseburia, Lactobacillus, Ruminiclostridium, Mucispirillum, and so on. This suggests that the combined intervention of DHF and MSFT primarily influenced the relative abundance of these genera, thus exerting its effects on the associated microbial community.

To identify biomarkers distinguishing between different abundance features and associated categories among groups, we employed LEfSe (linear discriminant analysis (LDA) effect size (LEfSe) analysis. The LEfSe tool identifies and interprets biomarkers in high-dimensional data sets, emphasizing both statistical significance and biological relevance of differences between groups.[51] In our analysis, we set the LDA threshold to 3 on the log scale, using the Kruskal–Wallis test with an α level of 0.05. Figures 6D and E show that Rheinheimera, uncultured bacteria, Pseudoalteromonadaceae, Pseudoalteromonas, Alteromonas, and Alteromonadaceae were abundantly different in the gut of saline-treated mice. Dechloromonas is the species that showed notable differential abundance in the group induced by Aβ oligomers. In the Aβ plus DHF group, significantly differentially abundant species include Rikenellaceae RC9 gut group, Ruminococcaceae UCG_014, Eggerthellaceae, Coriobacteriia, Coriobacteriales, Ruminococcaceae UCG_010,

Figure 6 Differences in microbiota among mice belonging to different groups according to the 16S rRNA data. (all groups: n=6) (**A** and **B**) Comparison of the distribution of microbiota at the phylum and genus levels across various groups. (**C**) Clustering chart of species abundance at the genus level. (**D** and **E**) Distribution histogram of LDA score and cladogram among different groups.

Ruminococcaceae UCG_005, and Ruminococcus_torques group, all belonging to Rikenellaceae RC9 gut group, Eggerthellaceae, and Ruminococcus_torques group. In the Aβ plus MSFT group, the significantly differentially abundant species include Epsilonbacteraeota, Helicobacter, Campylobacteria, and Helicobacteraceae. In the Aβ plus DHF and MSFT group, the significantly differentially abundant species include Bacteroides, Bacteroidaceae, Ruminococcaceae NK4A214 group, and Erysipelatoclostridium.

Microbial Variance Analysis in Response to Combined DHF and MSFT Intervention for Aβ-Induced Cognitive Impairments and Anxiety/Depressive Behaviors

The findings from behavioral studies indicated that the combined intervention of DHF and MSFT yielded significant improvements in cognitive impairments as well as in anxiety and depressive behaviors induced by Aβ. However, the effect of improving cognition was not significant with only DHF or MSFT. Notably, DHF alone demonstrates efficacy in ameliorating depression-like behaviors, whereas MSFT alone is effective in alleviating depressive and anxiety-like behaviors in Aβ mice. Consequently, our investigation focused on discerning the microbial variances among groups receiving DHF only, MSFT only, or the combined intervention. We conducted LEfSe analysis with an LDA threshold of 3. The findings revealed significant increase in Helicobacteraceae, Campylobacterales, and Acidobacteria in the Aβ plus MSFT group compared to the Aβ plus DHF plus MSFT group. Conversely, Erysipelatoclostridium, Eggerthellaceae, Parasutterella, and Pelomonas exhibited significant decrease in the Aβ plus MSFT group compared to the Aβ plus DHF plus MSFT group (Figure 7B). Similarly, LEfSe analysis was conducted on the Aβ plus DHF group and the Aβ plus DHF plus MSFT group. The results revealed that in comparison to the Aβ plus DHF group, Candidatus Arthromitus, Clostridiaceae1, and Eubacterium_coprostanoligenes group exhibited significant increase in the Aβ plus DHF plus

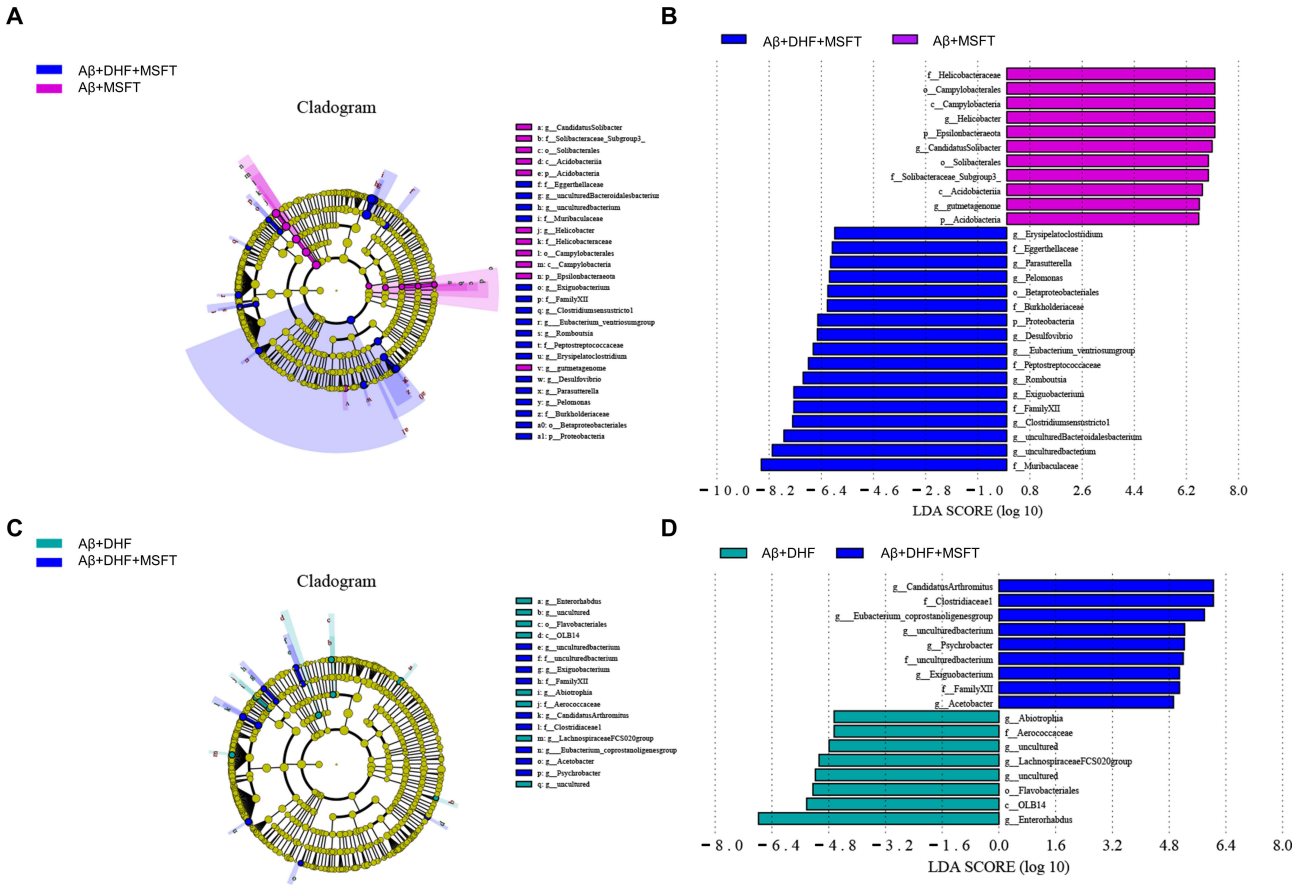

Figure 7 Distribution histogram of LDA score and cladogram. (**A** and **C**) The hierarchical tree diagram depicting the multi-level species hierarchy. (**B** and **D**) The LDA discriminant result plot of the differentially enriched gut microbiota between Aβ+DHF+MSFT Group and Aβ+DHF group or Aβ+MSFT group, with an LDA score >3.

MSFT group. In contrast, Abiotrophia, Aerococcaceae, Uncultured, and LachnospiraceaeFCS020 group experienced significant reductions (Figure 7D). The hierarchical tree diagram depicting the multi-level species hierarchy from the LEfSe analysis is presented in Figure 7A and C.

DHF and MSFT Intervention Leads to Different Composition of Metabolites in the Serum of Aβ-Induced Mice

The microbiome regulates the host metabolism via serum metabolites.[52] Hence, to detect the modified metabolites influenced by DHF and MSFT treatments, we collected serum from each group of mice, with each comprising four replicates. PCA revealed significant differences in serum metabolites between the Aβ group with interventions from DHF and MSFT compared to the untreated Aβ group (Figure 8A). In addition, the MA map highlighted metabolites that were expressed differently in Aβ oligomer-induced mice under DHF and MSFT interventions compared with those without interventions. Red and blue dots represent upregulated and downregulated metabolites, respectively (Figure 8B). Furthermore, we found that 77 distinct metabolites were clustered hierarchically within each sample. We selected the top 30 different metabolites for clustering analysis (Figure 8C). In addition, 25 distinct metabolites were clustered hierarchically between Aβ plus DHF group and DHF combined MSFT group. We found that the combined intervention group showed an increase in the expression of amino acid metabolism-related substances, including 3-Carboxypropyl_trimethylammonium cation, N-omega-Hydroxyarginine, D-Proline, and L-Pipecolic acid. Furthermore, there was an increase in the expression of substances related to neuro transmission and energy metabolism, such as Choline and Phosphocreatine. However, the expression of certain fatty acids or metabolites involved in fatty acid metabolism, such as alpha-Linolenic acid, Dihomo-gamma-Linolenic Acid, Stearoylcarnitine, 2-Methylbutyroylcarnitine, and 1-Palmitoyllysophosphatidylcholine, decreased (Figure 8D). Furthermore, we performed clustering analysis on the top 30 different metabolites in the Aβ plus MSFT group and the combined intervention group. The results showed that in the combined intervention group, there was an increase in the expression of metabolites such as Creatine, L-Tryptophan, Glycocholic acid, DL-lactate, Stearoylcarnitine, and Glycerophosphocholine. Conversely, the expression of metabolites such as Urea, N-omega-Hydroxyarginine, Myristic acid, 2-Hydroxy-3-methylbutyric acid, Creatinine, and D-Glucuronate decreased (Figure 8E). A comparison of the metabolite expression profiles between mouse models indicated that these modified metabolites might be potential AD biomarkers before and after treatment. KEGG analysis revealed that the abnormal metabolites were significantly concentrated in lipid metabolism (protein digestion and absorption and pyruvate metabolism), amino acid metabolism (glycine, serine, and threonine metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; and phenylalanine and tryptophan metabolism), and neurotransmitter metabolism (GABAergic synapse) (Figure 8F). Collectively, our findings indicate that the DHF and MSFT produces metabolites that have a significant impact on the pathways involved in the treatment of AD.

Gut Microbiota and Serum Metabolites are Correlated

Spearman correlation analyses were conducted to determine the association between the gut bacteria and serum metabolites. We analyzed the metabolic association heatmap and found that, at the genus level, there were correlations between the levels of gut microbiota and serum metabolites (Figure 9A). The correlation coefficient (r) and P-value are displayed for each cell in the hierarchical clustering heatmap. The correlation coefficient (r) is represented by a color: a positive correlation ($r > 0$) is shown in red, whereas a negative correlation ($r < 0$) is shown in green. Darker colors indicate a stronger correlation because the intensity of the color reflects the strength of the correlation. Correlations are given a P-value to indicate their significance. In the Aβ-induced mice, there was a significant increase in Bacteroides and Ruminococcaceae, as observed in the Aβ plus DHF and MSFT group (Figure 6C). Heatmap analyses revealed that Bacteroides showed a positive relationship with metabolites, such as N-omega-hydroxyarginine, DL-Phenylalanine, Ergothioneine, and others. However, it was negatively associated with metabolites such as DL-Indole-3-lactic acid, 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine, Dihomo-gamma-Linolenic Acid, which exhibited positive associations with amino acid metabolism and neurotransmitter synthesis and negative associations with lipid metabolism and inflammatory response (Figure 9A). Ruminococcaceae exhibited a favorable association with substances, such as

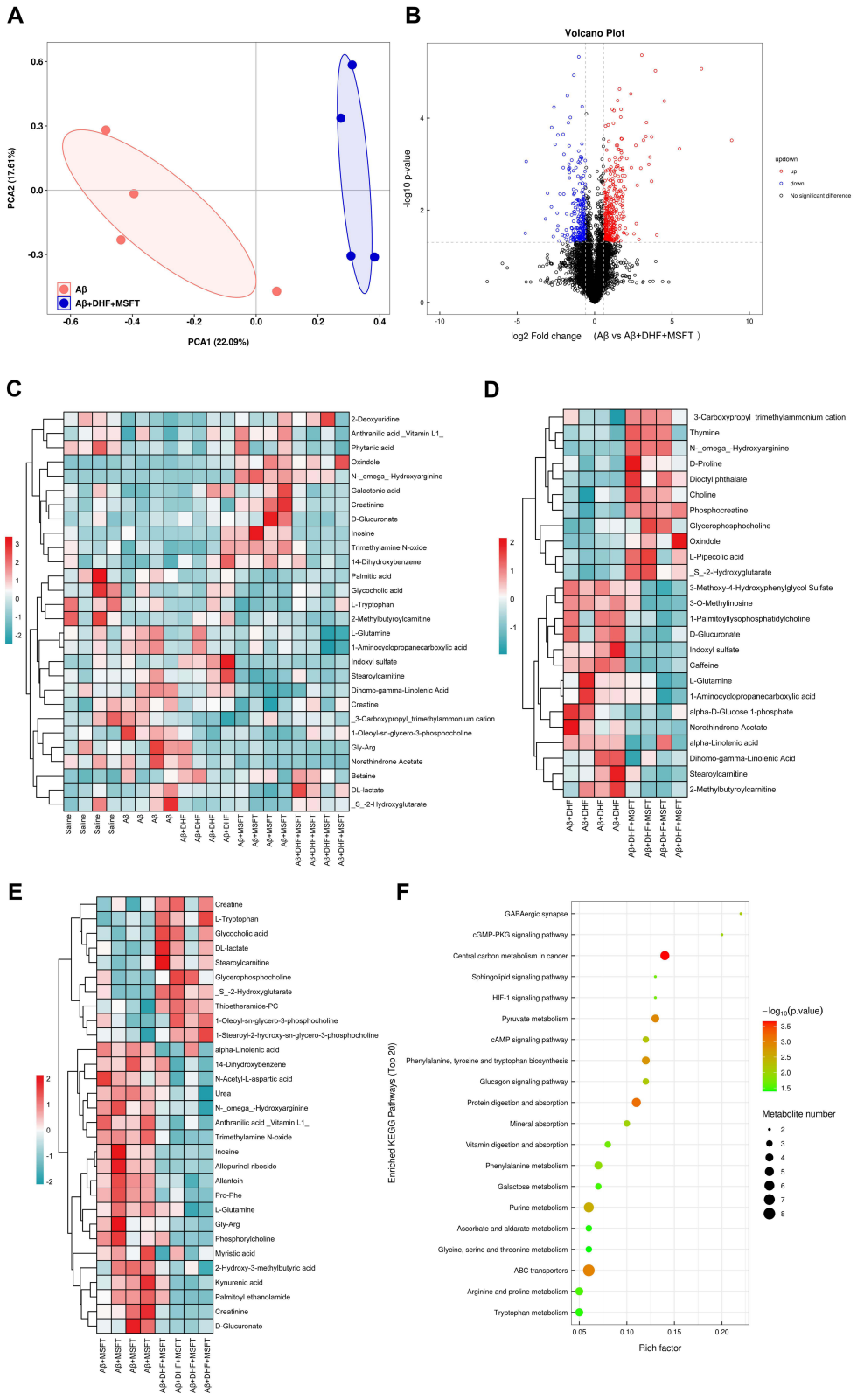

Figure 8 DHF and MSFT intervention altered serum metabolite expression profiles in mice. (**A**) PCA is employed to assess the metabolite of Aβ group and Aβ + DHF + MSFT group (all groups: n=4). (**B**) An MA map illustrates the metabolites that were differentially expressed in Aβ + DHF + MSFT mice compared with that in mice induced by Aβ oligomers. (**C**–**E**) A heat map depicting the hierarchical expression profile of different metabolites. (**F**) Between DHF and MSFT interventions, differentially expressed metabolites are identified using GO analysis.

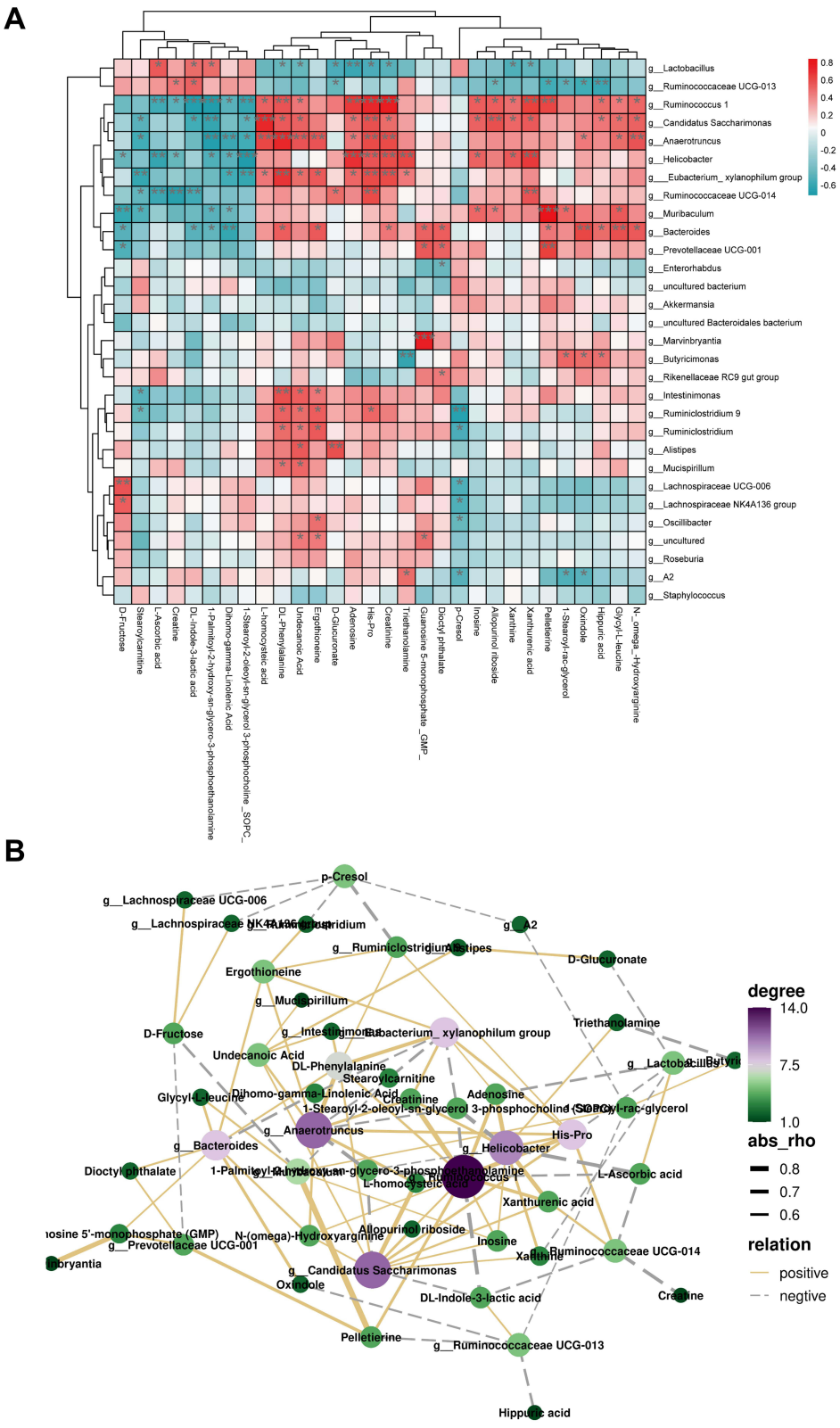

Figure 9 Analysis of Spearman correlation between microbiota and metabolites. (**A**) Clustering heatmap of microbiome and metabolites at the genus level. The heatmap displays a total of 166 significantly correlated differential genera and metabolites, with 54 pairs exhibiting a more significant correlation with P < 0.01. (**B**) Network diagram depicting the correlation between microbiome and metabolites in each group. The network displays the correlation between 30 metabolites and 30 bacterial genera. *P < 0.05, **P < 0.01 and ***P < 0.001.

His-Pro, L-homocysteic acid, DL-Phenylalanine and others. However, it displayed an unfavorable correlation with substances such as Dihomo-gamma-Linolenic Acid, 1-Stearoyl-2-oleoyl-sn-glycerol 3-phosphocholine (SOPC) and Stearoylcarnitine. These substances demonstrated positive correlations with amino acid metabolism and neurotransmitter synthesis but negative correlations with energy and lipid metabolism (Figure 9A). Helicobacter exhibited a favorable association with metabolites such as creatinine and inosine, but an unfavorable association with metabolites such as L-Ascorbic acid, stearoylcarnitine, 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine and so on. These metabolites were positively correlated with energy metabolism and negatively correlated with neurotransmitter synthesis and the inflammatory response (Figure 9A). The treatment with DHF and MSFT had a noticeable impact on the proportions of Ruminococcaceae and Bacteroides, leading to a reduction in the proportion of Helicobacter. Thus, this alteration can potentially impact amino acid and lipid metabolism, neurotransmitter synthesis, and inflammation in mice induced by Aβ oligomers.

Network analysis was conducted using Spearman correlation to identify important nodes in the network corresponding to these significant entities. The analysis focused on bacterial genera and metabolites that had correlation coefficients falling within the range of [0.5, 1] in absolute terms and a P-value below 0.05. As shown in Figure 9B, we performed a correlation analysis of 30 metabolites and 30 bacterial genera. The lines connecting them represent the nature of the correlation, with dotted lines indicating a negative correlation and solid lines indicating a positive correlation. Furthermore, the correlation coefficient is directly proportional to the thickness of the lines. The size of each node corresponds to its degree, meaning that nodes with higher degrees are larger. In this network, the central nodes of the metabolites with higher degrees were His-Pro, DL-Phenylalanine, Ergothioneine, Adenosine, and Creatinine. Metabolites were strongly connected to pathways involved in inflammation, synthesis of proteins and neurotransmitters, and energy metabolism. The central nodes of the bacterial genera were Ruminococcus, Candidatus Saccharimonas, Helicobacter, and Bacteroides. These findings indicated that these core metabolites and bacterial species could potentially be the primary focus for DHF and MSFT in impacting cognitive impairment, anxiety-related, and depression-related symptoms in mice induced with Aβ oligomers.

Discussion

AD is the primary neurodegenerative condition responsible for memory loss, cognitive decline, and behavioral issues in the older population.[53] The etiology and pathogenesis of AD are multifaceted, but toxic Aβ oligomers are considered a crucial element. Prior research has shown that introducing Aβ oligomers into the lateral ventricles or hippocampus of mice, rats, or macaques could trigger AD-like pathological and behavioral features. These experiments emphasize the capacity of Aβ oligomers to trigger an animal model of AD accompany depression and anxiety.[54] In this study, we created Aβ oligomers from Aβ monomers and administered them to both sides of CA1 region in ICR mice hippocampus to establish the AD model. After the administration of Aβ oligomers, we witnessed significant cognitive decline and observed signs of anxiety and depression in the mice. These findings indicate that the changes in neurobehavioral impairments caused by Aβ oligomers accurately replicate the behavioral traits linked to AD accompany depression and anxiety. Currently, pharmacotherapy remains the mainstream approach for the treatment of AD; however, because of its constraints, alternative approaches, such as cognitive enhancement, bodily activity, and sensory engagement, have garnered growing interest in mitigating and enhancing AD.[50,55,56] A wealth of evidence indicates that exercise interventions can reduce Aβ deposition, enhance neural plasticity, stimulate neurogenesis, improve cognitive function, enhance daily living abilities, and decrease the incidence of neuropsychiatric symptoms.[57,58] For instance, exercise increases the expression of BDNF, a dose-dependent regulator of hippocampal neurogenesis, which ameliorates cognitive dysfunction. Substantial evidence suggests that the positive effects of physical activity on neuroplasticity and resilience to cellular stress are associated with BDNF. Moreover, physical activity and BDNF have the potential to increase the breakdown of AβPP by α-secretase in nerve cells, resulting in the generation of secreted AβPPα (sAβPPα). sAβPPα, in turn, can inhibit β-secretase, thereby preventing the generation of neurotoxic Aβ and its neuronal toxic effects. Therefore, it remains unknown whether exercise and BDNF act together to reduce the risk of AD, or its effect on anxiety-depression-like behaviors.[59,60]

Acousto-optic stimulation has been suggested to decrease the pathology and cognitive impairments associated with AD by enhancing the transmission of signals through gamma oscillations. Individuals with AD experience disruptions in the neural circuits responsible for gamma oscillations, which play a role in higher cognitive functions and have a frequency range of 30–90 Hz.[61] Moreover, studies have shown that when auditory and visual stimulation are combined at a frequency of 40 Hz, they can induce gamma oscillations and alleviate AD-related pathology by decreasing amyloid plaques, thereby enhancing cognition.[26] Recent studies in animals and clinical settings have shown that visual stimulation at a frequency of 40 Hz results in increased production of cytokines and microglial chemokines. This impact extends to neural networks within the nervous system, as well as various immune factors. These findings suggest the potential therapeutic benefit of this treatment for neuroinflammation.[62] Our previous findings suggested that multifaceted intervention could ameliorate AD symptoms by promoting nerve regeneration,[10] however, it remains unclear whether this intervention operates through BDNF or whether modulating BDNF expression influences its neuroregulatory role in AD with depression and anxiety.

In the present study, we employed DHF as a surrogate for BDNF expression. DHF, a natural flavonoid compound and TrkB agonist mimics the effects of endogenous BDNF on TrkB activation.[63] It is widely used as an activator of the BDNF-TrkB pathway. Studies have revealed that DHF can simulate the neuroprotective effects of BDNF by activating TrkB, thereby promoting neuronal survival and development. Additionally, it enhances synaptogenesis and plasticity, thereby positively influencing learning and memory capabilities.[64] Therefore, we jointly applied DHF and MSFT to further explore the synergistic mechanism of regulating BDNF expression and combining MSFT on Aβ-induced mice. By combining these two interventions, we hypothesized that we could potentially achieve a more pronounced and comprehensive improvement in AD-related cognitive impairments. Our behavioral experiment findings demonstrate that the concurrent administration of DHF and MSFT for 4 weeks exhibits advantageous effects in enhancing cognitive ability and alleviating anxiety–depressive-like behaviors in Aβ mice. In our learning and memory experiments, such as NOR, YM, and MWM tests, we observed that neither DHF nor MSFT alone significantly improved cognition in the Aβ mice. However, when administered alone, MSFT did show a significant improvement in anxiety-like behavior as measured by EPM test. On the other hand, DHF alone improved both depression-like behavior, as assessed by TST and FST, and anxiety-like behavior in the Aβ mice. These results suggest that DHF is more associated with depression and MSFT is more associated with depression and anxiety. In summary, the joint intervention with DHF and MSFT proves to be a successful strategy for mitigating behavioral impairments, such as cognitive decline, anxiety, and depression, during the progression of AD.

BDNF, which is regarded as a pivotal controller of neural circuitry development and functioning, plays a significant role in neuronal differentiation, growth, synaptogenesis, synaptic plasticity, and higher-level cognitive processes.[65] Studies have shown that VGF enhances hippocampal neurogenesis and neurotrophic activity.[66,67] In addition, it has been found that BDNF regulates VGF expression and secretion. Moreover, the collective action of BDNF and VGF may work synergistically to enhance neuronal viability and facilitate synapse formation, thereby fostering neuronal growth and promoting connectivity.[68] PSD-95 plays a vital role in neuronal synapses. As a structural protein, PSD-95 is primarily found in the dense regions of excitatory post-synaptic membranes and contributes to synaptic formation, stability, and functional modulation. A decrease in PSD-95 is strongly associated with deterioration of synaptic plasticity and a decline in cognitive abilities.[69] Nrf2 plays a crucial role as a transcription factor in the regulation of cellular oxidative stress. It is involved in maintaining the balance of reactive oxygen species (ROS) within cells.[70,71] On the other hand, the activation state of GSK3β is closely associated with neural development and plays a significant role in various cellular processes, and p-CREB plays a vital role in memory and learning processes. Studies have suggested that the function of p-CREB is strongly linked to long-term potentiation (LTP) and synaptic plasticity, which play vital roles in the processes involved in memory creation and preservation.[3] The present investigation revealed that the DHF and MSFT combined treatment led to a substantial increase in the concentrations of BDNF, VGF, PSD-95, Nrf2, p-Ser9-GSK3β and p-CREB proteins in the hippocampus of Aβ-induced mice. However, DHF alone can only increase the expressions of BDNF, PSD95 and Nrf2, while MSFT alone can only increase the expressions of BDNF and VGF. This finding suggests that the combined intervention works synergistically to protect neurons from the reduced levels of proteins and transporters caused by Aβ oligomers. Furthermore, this result indicated that the combined intervention regulated multiple

crucial elements of the BDNF/VGF/CREB signaling pathway. Taken together, our findings suggest that the combined intervention approach could potentially modulate proteins associated with synaptogenesis, neurons, and signal transmission via the BDNF/VGF/CREB signaling pathway to promote improvements in behavioral performance.

To investigate the complex interactions between the gut microbiota and serum metabolites, we performed metabolomics on multiple compartments and 16S rRNA sequencing. The results indicated a notable disparity in the gut microbial makeup between mice induced with Aβ oligomers and mice treated with saline. Akkermansiaceae, Bacteroidaceae, and Deferribacteraceae exhibited lower abundance in Aβ-induced mice than in saline-treated mice, whereas Staphylococcus showed higher abundance in Aβ-induced mice than in saline-treated mice. Moreover, Akkermansia, Prevotellaceae UCG-001, Bacteroides, Butyricicoccus, Ruminococcaceae, Alistipes, Roseburia, Lactobacillus, Ruminiclostridium 5, Blautia, and Desulfovibrio in the Aβ oligomer plus DHF and MSFT group were more abundant than those in the Aβ oligomer-induced group. We observed that Helicobacteraceae, Campylobacterales, and Acidobacteria were more abundant in the Aβ plus MSFT group than in the Aβ plus DHF plus MSFT group. In contrast, the abundance of Erysipelatoclostridium, Eggerthellaceae, Parasutterella, and Pelomonas decreased significantly. Candidatus Arthromitus, Clostridiaceae1, and Eubacterium_coprostanoligenes group exhibited significant increases in the Aβ plus DHF plus MSFT group compared to the Aβ plus DHF group. But the abundance of Abiotrophia, Aerococcaceae, Uncultured, and LachnospiraceaeFCS020 group decreased. These findings were comparable with those of previous studies, suggesting a potential correlation between these specific groups of bacteria and the development and advancement of AD.[72,73] After applying the multifactor intervention, we identified 77 abnormal metabolites expressed in these groups. Among these metabolites, the levels of Creatine, L-Tryptophan, Glycocholic acid, DL-lactate, Stearoylcarnitine, and Glycerophosphocholine were elevated. Among them, the metabolic profiles of Aβ plus DHF group and DHF combined with MSFT group were mainly different in amino acid metabolism, neurotransmitter, energy metabolism and fatty acid metabolism. The metabolic differences between the Aβ plus MSFT group and the combined intervention group were mainly related to the metabolic pathways of energy metabolism, neurotransmitter synthesis and signal transduction. Notably, the combined intervention led to increased expression of metabolites associated with amino acid metabolism and neurotransmitters, while expression of certain fatty acids and metabolites involved in fatty acid metabolism decreased. KEGG analysis revealed that abnormal metabolites were significantly associated with lipid metabolism (protein digestion and absorption and pyruvate metabolism), amino acid metabolism (glycine, serine, and threonine metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; phenylalanine metabolism; and tryptophan metabolism), and neurotransmitter metabolism (GABAergic synapse). Several studies have shown that individuals with AD exhibit disturbances in amino acid metabolism that affect neuronal function, cognition, and memory impairment.[74] Furthermore, research has shown a direct correlation between exercise intensity and blood lactate levels, with a notable increase in lactate concentration observed during endurance activities.[75] To enhance our understanding of the role of gut microbiota in AD pathogenesis, we conducted a joint gut-serum metabolomics analysis to investigate in vivo metabolic alterations. The findings indicated that the use of DHF and MSFT had a substantial impact on the proportion of Ruminococcaceae and Bacteroides, leading to a reduction in the proportion of Helicobacter. These alterations can impact amino acid and lipid metabolism, neurotransmitter synthesis, and inflammatory response in mice induced by Aβ oligomers. Consequently, the microbiome and metabolites with varying abundances could potentially be targeted for therapeutic purposes in AD and could also function as potential biomarkers.

Conclusion

Our findings suggest that a multifactorial intervention involving both DHF and MSFT holds promise for alleviating psychiatric symptoms and cognitive impairment in mice induced with Aβ oligomers. Notably, the synergistic application of DHF and MSFT proves more effective in preventing the decline of immediate recollection, while also suppressing depressive and anxious manifestations amid Aβ oligomer-induced neurotoxicity, compared to interventions focused solely on one aspect. These findings highlighted the potential benefits of a comprehensive treatment strategy that addresses multiple factors simultaneously. Furthermore, insights from the analysis of gut bacteria and serum compounds suggested that Ruminococcaceae, Bacteroides, and Helicobacter significantly influenced the metabolism of amino acids and lipids, synthesis of neurotransmitters, and inflammatory response in Aβ oligomer-induced mice. Our research

suggests novel mechanisms through which the gut microbiota may impact serum metabolism, with indications that the BDNF/TrkB/CREB signaling pathway may contribute to the beneficial effects of DHF and MSFT. This provides new perspectives on the intricate interactions between the gut microbiota, metabolites, and AD.

Ethics Approval

This study was approved by the Animal Ethics and Welfare Committee (AEWC) of Ningbo University (Approval No. AEWC-2019-04).

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Disclosure

The authors report no conflicts of interest in this work.

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