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Mannan oligosaccharide attenuates cognitive and behavioral disorders in the 5xFAD Alzheimer's disease mouse model via regulating the gut microbiota-brain axis

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive deficits and psychiatric symptoms. The gut microbiota-brain axis plays a pivotal role during AD development, which could target nutritional intervention. The prebiotic mannan oligosaccharide (MOS) has been reported to reshape the gut microbiome and enhanced the formation of the neuroprotective metabolites short-chain fatty acids (SCFAs). Here, we found that an 8-week treatment of MOS (0.12%, *w/v* in the drinking water) significantly improved cognitive function and spatial memory, accompanied by attenuated the anxiety- and obsessive-like behaviors in the 5xFAD transgenic AD mice model. MOS substantially reduced the Aβ accumulation in the cortex, hippocampus, and amygdala of the brain. Importantly, MOS treatment significantly balanced the brain redox status and suppressed the neuroinflammatory responses. Moreover, MOS also alleviated the HPA-axis disorders by decreasing the levels of hormones corticosterone (CORT) and corticotropin-releasing hormone (CRH) and upregulated the norepinephrine (NE) expressions. Notably, the gut barrier integrity damage and the LPS leak were prevented by the MOS treatment. MOS re-constructed the gut microbiota composition, including increasing the relative abundance of *Lactobacillus* and reducing the relative abundance of *Helicobacter*. MOS enhanced the butyrate formation and related microbes levels. The correlation analysis indicated that the reshaped gut microbiome and enhanced butyrate formation are highly associated with behavioral alteration and brain oxidative status. SCFAs supplementation experiment also attenuated the behavioral disorders and Aβ accumulation in the AD mice brain, accompanied by balanced HPA-axis and redox status. In conclusion, the present study indicated that MOS significantly attenuates the cognitive and mental deficits in the 5xFAD mice, which could be partly explained by the reshaped microbiome and enhanced SCFAs formation in the gut. MOS, as a prebiotics, can be translated into a novel microbiota-targeted approach for managing metabolic and neurodegenerative diseases.

1. Introduction

Alzheimer's disease (AD), as a neurodegenerative disease, seriously threatens about 6% of people 65 years and older (Burns and Iliffe, 2009). Around 50 million people worldwide are suffering from dementia, and the total number of patients is expected to reach 131 million by 2050 (Tiwari et al., 2019; Van Cauwenberghe et al., 2016). AD patients suffer from memory loss and cognitive disorder accompanied by neuropsychiatric symptoms accelerating cognition impairment, such as anxiety and depression. (Ferretti et al., 2001; Ismail et al., 2016; Pietrzak et al., 2015). Nevertheless, the mechanisms of AD pathogenesis have remained largely abstruse. The main pathological changes are characterized by neurofibrillary tangles with excessive hyperphosphorylated Tau protein in nerve cells and senile plaques accumulated of aberrant extracellular amyloid-β (Aβ) (Arriagada et al., 1992; Janus et al., 2000). The accumulation of Aβ triggers inflammatory responses and oxidative

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stress in the brain, which are the main risk factors for dementia-related neurodegeneration (Butterfield et al., 2001; Dudal et al., 2004; Lue et al., 1996). Specifically, these hallmarks occur in the brain regions of the cerebral cortex and hippocampus, which is classically related to declining cognitive function (Manczak et al., 2018). Moreover, it has previously been found that the aggregation of Aβ in the prefrontal cortex (PFC) and amygdala, the essential emotion regulating regions, is positively associated with the anxiety-like behavior in AD transgenic mice and AD patients (Davis and Whalen, 2001; España et al., 2010; Ramakers et al., 2012). Besides, the dysfunction of the hypothalamic-pituitaryadrenal (HPA) axis in patients with AD has been reported in several studies (Buckley, 2008; Pietrzak et al., 2017). Thus, it is speculated that the above changes may affect neural activity disorders such as cognitive and emotional dysfunction due to they gradually emerged in different brain regions.

Although the pathogenesis of AD remains unclear, a growing body of evidence suggests that the microbiota-gut-brain axis plays a pivotal role in adjusting brain functions (Sampson and Mazmanian, 2015; Wang et al., 2019b). It has been found that the gut microbiome of AD patients is significantly altered compared with healthy individuals (Vogt et al., 2017). It has been reported that the administration of probiotics such as microbes of *Lactobacilli* prevented cognitive decline and anxiety in both humans and animal studies (Messaoudi et al., 2011; Savignac et al., 2014). The altered gut microbiota also appears to be associated with Aβ accumulation (Luca et al., 2019; Minter et al., 2016). Interestingly, it has also been demonstrated that *Lactobacillus* and *Bifidobacterium* can improve the HPA axis function and modulate the behavior stress response (Ait-Belgnaoui et al., 2018; Frankiensztajn et al., 2020). Therefore, microbiota composition changes were closely relevant to behavioral disorders and psychiatric disturbance. Targeting modulation of gut homeostasis may be feasible for cognitive impairment and mood dysfunction of AD.

Mannan oligosaccharide (MOS) is one of the prebiotics derived from Konjac and the outer cell-wall membrane of bacteria, plants, or yeast (Pangsri et al., 2015). Various studies have indicated that MOS treatment can markedly reduce inflammation, prevent obesity, and strengthen the immune system via its mediating effects on gut microbiota (Ferenczi et al., 2016; Hoving et al., 2018b; Wang et al., 2018). Our recent study showed that reshaped gut microbiota alleviated the cognitive deficits and anxious behaviors in a mouse model by generating beneficial microbial metabolites such as short-chain fatty acids (SCFAs) (Liu et al., 2020). Dietary supplementation of MOS could suppress the appetite and systemic insulin resistance of obese mice by reshaping the gut microbial composition and enhancing the formation of SCFAs (Yan et al., 2019). SCFAs are the end-products of prebiotic fermentation, speculated to act as bridges of the microbiota-gut-brain axis (Slavin, 2013). It has been reported that SCFAs treatment prevents neuronal injury by inhibiting neuroinflammation and exhibits behavior-specific antidepressant and anti-anxiety effects in animal models (Matt et al., 2018; van de Wouw et al., 2018). Here, we hypothesized that MOS supplementation could ameliorate behavioral deficits containing memory loss and anxiety during AD development by enhancing SCFAs formation.

The present research explored the beneficial effects of MOS on cognitive and behavioral disorders in 5xFAD-Tg mice, a classic transgenic AD mouse model (Oakley et al., 2006). The memory deficit and non-cognitive behavioral tests were conducted on the impact of MOS on cognitive impairment and emotionality disorders in AD mice. To detect the physiological indicators of the brain, the Aβ accumulation, neuroinflammatory response, oxidative damage, and HPA axis activity were investigated. Moreover, the gut microbiota and metabolites SCFAs were characterized. The correlation analysis between the butyrate levels and other biochemical indexes was conducted, and a SCFAs supplementation experiment was established to confirm the mediating roles of these microbial metabolites involved in the neuroprotective effects of the MOS treatment.

2. Materials and methods

2.1. Animals

The male 5xFAD-transgenic mice (Stock No: 006554) on a congenic C57BL6 background were provided by the Jackson Laboratory (Bar Harbor, ME, USA). The 5xFAD mice carry human amyloid precursor protein (APP) as well as the human presenilin 1 (PSEN1) transgenes with a total of five AD-related mutations, including the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP, and the M146L and L286V mutations in PSEN1. The 5xFAD mice periodically backcrossed generations with female C57BL6 wild-type stock (Stock No: 006554) from Jackson Labs. Mice were genotyped for both the APP and PS1 transgenes with polymerase chain reaction (PCR) using DNA samples obtained via ear tissue (DNA extraction Kit (centrifugal column), Beijing Betek Biotechnology Co. LTD, Beijing, China). Moreover, mice carried the $Pde6b^{rd1}$ allele, which is a recessive mutation and leads to retinal dysfunction. They were excluded from the study to ensure that visual impairment does not interfere with behavioral tests. In this study, mice were fed until 6 months old. Age-matched wild-type mice (six months old) on the same background were used as the controls. All animals were single-housed with a constant temperature of 22 ± 2 °C, relative humidity 55 ± 5% under a 12/12 h light–dark cycles (lights on at 8:00 a.m.) in the facility of Northwest A&F University.

2.2. Experimental design

The 5xFAD and wild-type (WT) mice were randomly divided into 6 groups (6 months old, n = 8 per group): WT group, WT + MOS group, WT + SCFAs group, 5xFAD group, 5xFAD + MOS group, and 5xFAD + SCFAs group. All MOS group mice were administered the prebiotic MOS (0.12% *w/v* in the drinking water, with a purity ~ 85%; Yuansen Biological Technology Ltd., Xi'an, China) or SCFAs mixture (acetate 67.5 mM, propionate 40 mM, butyrate 25 mM; Yuanye Biological Technology Ltd., Shanghai, China) for 8 weeks. The dosage of MOS in the current study was chosen according to previous experiments and related references (Asano et al., 2004; Hoving et al., 2018a), and the concentration of SCFAs was chosen based on the previous literature (Smith et al., 2013; Tian et al., 2018). The mice in the vehicle group were fed with normal drinking water. All animals were kept and subjected to a standard diet (AIN-93 M, TROPHIC Animal Feed High-Tech Co., Ltd., Nantong, China) and were *ad libitum* fed. The body weight and food intake were measured and recorded weekly. The drinking water added MOS or SCFAs was replaced twice a week and recorded water intake. All experimental protocols followed guidelines for the care and use of laboratory animals: Eighth Edition (ISBN-10: 0–309-15, 396–4). The animal experiment program was approved by the Animal Ethics Committee of Northwest A&F University and BGI Institutional Review Board on Bioethics and Biosafety (BGI-IRB).

After behavior tests, mice feces were collected in a sterile environment, and the mice were sacrificed under anesthesia to minimize the suffering. The blood samples collected from the orbit were centrifuged to obtain the serum and stored at −80 °C for subsequent examination. The brain, viscera, and gut tissues were collected, which were rapidly frozen with liquid nitrogen or preserved with 4% paraformaldehyde for further biochemical determination.

Other detailed methods, including *Behavioral tests*, *ELISA assay and LPS content*, *Oxidative stress index*, *SCFAs levels analysis*, *qRT-PCR analysis*, *Hematoxylin and eosin (H&E) staining*, *Immunohistochemical staining and immunofluorescence staining*, and *16S rRNA Microbiome sequencing*, can be found in the **Supplementary Information**.

2.3. Statistical analysis

R software (version 3.6.1), SPSS 22, and Graphpad Prism 7.0 (GraphPad Software Inc, San Diego, CA, USA) was used for the data

process. The body weight gain, food intake, water intake, behavioral indexes, PCR, quantification of section staining, kit tests, and SCFAs content were analyzed by two-way ANOVA, followed by the Tukey's test for post-hoc analysis. The Wilcoxon-signed rank test was used to compare gut microbiome composition among different groups for beta-diversity based on the unweighted UniFrac phylogenetic distance. Spearman correlation analysis was used to conduct the correlations among the butyrate in feces and serum levels and other biochemical indexes, including NE, CORT, MDA, the relative abundance of microbiota, and behavior tests. The data were represented by mean ± SEM, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of MOS treatment on cognitive impairments in 5xFAD mice

The animal genotype identification and experimental workflow were conducted as described in the **Methods & materials** section and as shown in **Fig. S1A and 1A**. During the experiment, there was no significant effect of genotype and MOS, as well as no interaction effect on body weight gain, food intake, and water intake, suggesting that the MOS treatment had no effects on the energy intake and body weight changes in the AD mice (Fig. 1**B-E**).

To investigate the protective effects of MOS on the cognitive function in the AD mice, a series of behavioral tests, including the MWM test and Y-maze test conducted. On the 5th day of place navigation in MWM test, two-way ANOVA analysis showed a significant effect of genotype [F (1,

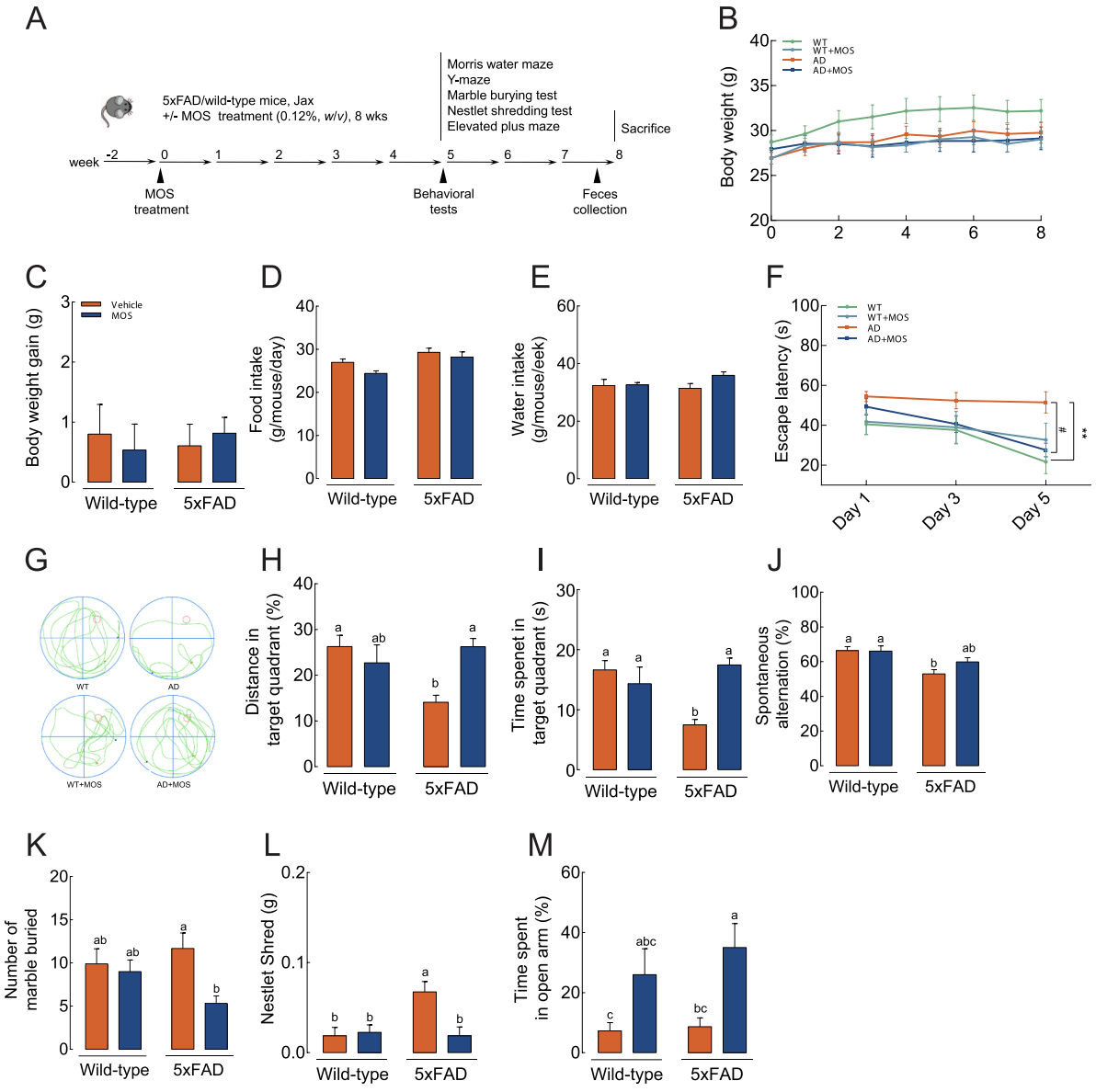

Fig. 1. Effects of MOS Treatment on Cognitive Impairments and Behavioral Disorders in 5xFAD Mice. (A) The experimental workflow of animal treatments (n = 8). **(B)** Body weight and **(C)** body weight gain during the 8 weeks treatment. **(D)** Food intake. **(E)** Fluid intake of mice; The cognitive function and spatial memory were evaluated by the Morris water-maze test (MWM). **(F)** Escape latency, **(G)** representative track of the test on day 6, **(H)** the proportion of distance in the target quadrant (%), **(I)** the time in the target quadrant (s); The working memory was evaluated by the Y maze test. **(J)** Spontaneous alternation (%); The mental disorders were evaluated by the marble-burying test (MBT), the nestlet shredding test (NST), and the elevated plus-maze test (EPM). **(K)** Number of marble buried in the MBT, **(L)** nestlet Shred (g) in the NST, and **(M)** percentage of time in open arm (%) in the EPM. Data are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test. The letters (a – c) indicated significant differences among all treatment groups ($p < 0.05$).

28) = 4.251, P = 0.0486], and a significant interaction effect between genotype × MOS [F (1, 28) = 8.407, P = 0.0072] on escape latency. There was no significant effect of MOS. Post-hoc analysis showed that there was a dramatic difference between wild-type (WT) mice and 5xFAD (AD) mice (Fig. 1**F**) ($p < 0.01$), indicating that AD mice exhibited cognitive and spatial memory loss. However, the MOS treatment significantly decreased the escape latency in AD mice but not in the WT mice (Fig. 1**F**) ($p < 0.05$). On the 6th day, the platform was removed for the probe trial. The results showed a significant interaction effect between genotype × MOS [F (1, 28) = 9.173, P = 0.0052] on distance in the target quadrant. A significant effect of MOS [F (1, 28) = 4.833, P = 0.0363] and an interaction between genotype × MOS [F (1, 28) = 12.33, P = 0.0015] on time spent in the target quadrant were also detected. Post-hoc analysis showed that AD mice swam less distance (Fig. 1**H**) ($p < 0.05$) and spent less time in the target quadrant than WT mice (Fig. 1**I**) ($p < 0.01$). However, MOS treatment prominently enhanced the residence distance (Fig. 1**H**) ($p < 0.05$) and traveling time (Fig. 1**I**) ($p < 0.01$) in the target quadrant of AD mice, which indicated that MOS improved cognitive deficits of AD mice.

To further explore the effects of the MOS on the working memory in the AD mice, the Y-maze test was conducted. A significant effect of genotype [F (1, 28) = 14.62, P = 0.0007] was also observed, but there was no significant effect of MOS and interaction between genotype × MOS. The spontaneous alteration was significantly lower in the AD group than the WT group (Fig. 1**J**) ($p < 0.01$), which suggested that the working memory is impaired in AD mice. However, compared with the vehicle group, the MOS treatment had no significant improvement effects on working memory in AD mice (Fig. 1**J**). These results illustrated that the MOS treatment significantly improved the cognitive and spatial memory loss, but not the working memory impairment in AD mice.

3.2. Effects of MOS treatment on anxiety-like behaviors in 5xFAD mice

To determine the psychological behavioral changes in AD mice, the NST, MBT, and EPM were performed in the current study. Buried bead behavior is a manifestation of instinctive anxiety-like and compulsive behavior in rodents (Dixit et al., 2020; Njung'e and Handley, 1991). Two-way ANOVA analysis showed a significant effect of MOS [F (1, 28) = 6.067, P = 0.0202], but no significant effect in genotype [F (1, 28) = 0.2603, P = 0.6139], as well as interaction effect between genotype × MOS [F (1, 28) = 2.752, P = 0.1083]. Although the number of buried beads in AD mice was not significantly higher than that in WT mice, marble burying was markedly inhibited by MOS treatment in AD mice (Fig. 1**K**) ($p < 0.05$). As for the NST, a two-way ANOVA showed a significant effect of genotype and MOS [F (1, 28) = 5.281, P = 0.0292; F (1, 28) = 5.281, P = 0.0292], and a significant genotype × MOS interaction [F (1, 28) = 7.187, P = 0.0122] on nestlet shredding. Post hoc analysis showed that AD mice shredded more cotton than WT mice (Fig. 1**L**) ($p < 0.01$), indicating that AD mice had anxious and compulsive-like behaviors. However, MOS treatment significantly reduced the nestlet shred in the AD group (Fig. 1**L**) ($p < 0.01$). EPM is a well-recognized behavioral test for anxiety-like behavior evaluation (Rodgers and Dalvi, 1997). The anxiety-like behavior of the animal is examined by the time spent in the open arm (Pellow et al., 1985). Therefore, EPM was used to test anxiety levels in 5xFAD mice. It was found that there was no significant effect of genotype and interaction effect on the percentage time spent in the open arm. However, we observed a significant effect of MOS [F (1, 28) = 13.21, P = 0.0011]. We found that MOS significantly elevated the percentage time spent in open arms in AD mice, which reveals preventive effects on anxiety-like behavior in the AD mice (Fig. 1**M**) ($p < 0.05$). These data indicated that MOS attenuated the behavioral disorders in AD mice.

3.3. Effects of MOS treatment on histopathological changes and amyloid deposition of brain in 5xFAD mice

The neuronal morphological changes in mice brain tissues were conducted by the H&E staining. As shown in Fig. 2**A**, the nucleus was severely condensed, and shrinking neurons were found in PFC, hippocampal CA1 region, and amygdala in the AD mice brain. MOS could prevent the mentioned above histopathological damages and restore to normal.

The deposition of Aβ is one of the neurological hallmarks of AD (Storey and Cappai, 1999). In this study, immunofluorescence staining was employed to evaluate amyloid deposition in mice brains (Fig. 2**B**). AD mice assembled more plaques in their brains than WT mice (Fig. 2**C-E**) ($p < 0.01$). However, the elevation of the amyloid-positive area was significantly decreased by MOS treatment, especially in the hippocampal CA1 and amygdala (Fig. 2**D-E)** ($p < 0.01$). Consistently, the mRNA expressions of amyloid precursor protein (APP) and β-secretase (Bace1) in the cortex and hippocampus of AD mice were higher than the WT mice (Fig. 2**F-I**) ($p < 0.01$), while MOS remarkably declined both genes' expressions (Fig. 2**F-I**) ($p < 0.01$), indicating the potential role for reducing Aβ deposition by MOS. Therefore, MOS treatment improved neuronal morphology and reduced the accumulation of Aβ in AD mice brain.

3.4. Effects of MOS treatment on inflammatory responses and oxidative stress of brain in 5xFAD mice

Here, we found that the MDA content significantly higher in the AD mice cortex than in WT mice (Fig. 3**A**) ($p < 0.01$), while MOS treatment substantially reduced the MDA levels of AD mice brain (Fig. 3**A**) ($p < 0.01$). The level of GSSG in AD mice brian dramatically higher than WT mice, which was statistically reduced by MOS treatment (Fig. 3**B**) ($p < 0.05$). Additionally, the GSSG/GSH was significantly reduced by the MOS treatment in the cortex of AD mice, but not in that of WT mice (Fig. 3**C**) ($p < 0.01$). The same results were obtained in immunohistochemical staining of 8-OHdG, a biomarker of oxidized DNA damage. As shown in the graphs (Fig. 3**D**), the AD mice reported significantly higher 8-OHdG levels than the WT mice in the different brain areas (Fig. 3**E-G**) ($p < 0.05$). Compared with the vehicle, MOS treatment significantly reduced the levels of 8-OHdG levels in AD mice brain, especially in the PFC (Fig. 3**E**) ($p < 0.05$).

Relevant studies have shown that excessive oxidative damages promote forming a cascade reaction to expand inflammation (Steele et al., 2007). It is known that loss of homeostasis in microglia leads to neurodegenerative disease, especially in AD (Spangenberg and Green, 2017). Here, it has been found that the positive area of Iba-1, a biomarker of activated microglia, was significantly increased in the PFC, hippocampus, and amygdala in AD mice compared with the WT mice (Fig. 3**H-K**) ($p < 0.05$). MOS significantly reduced the Iba-1 positive cells in these regions compared with the vehicle-treated AD mice (Fig. 3**H-J**) ($p < 0.05$). Moreover, the graph showed that the majority of microglia in the brain of AD mice were amoeboid type, whereas the microglia were converted into the ramified type by MOS treatment (Fig. 3**H**), which indicated that MOS could reduce the microglial over acitviation. Furthermore, MOS treatment decreased the overexpression of TNF-α and IL-6 in AD mice brain (Fig. 3**L** and **M**) ($p < 0.05$). Overall, these results showed that MOS supplementation effectively alleviated neuroinflammation and oxidative damage in the AD mice brain.

3.5. Effects of MOS treatment balanced hormonal dysfunction of HPA axis and norepinephrine in 5xFAD mice

The chronically active HPA axis leads to neuronal damage and death, thus enhancing vulnerability to neurodegenerative diseases such as AD (Herman and Seroogy, 2006). To evaluate the homeostasis of the HPA axis, the levels of hormones CORT and CRH were measured. The data

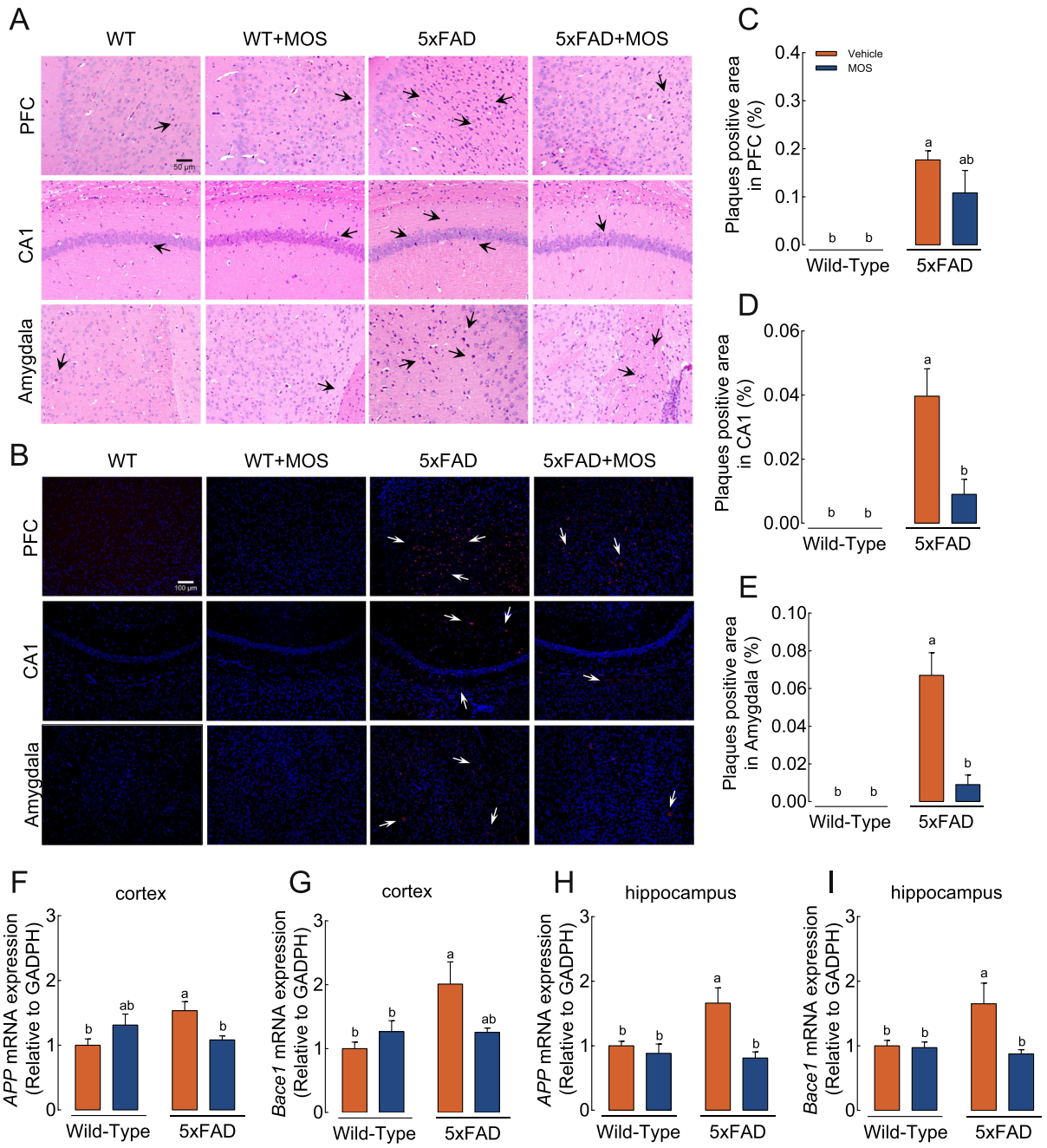

Fig. 2. Effects of MOS Treatment on Histopathological Changes and Amyloid Deposition of Brain in 5xFAD Mice. (A) Representative images of H&E staining of different regions in the brain. **(B)** Representative images of immunofluorescence staining of different regions in the brain. **(C-E)** Quantification of amyloid deposition area based on immunofluorescence staining sections by ImageJ software (Representative images were captured from 6 slices of 3 mice per group). **(F-I)** The mRNA expression of APP and Bace1 in cortex and hippocampus (n = 4). Data are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test. The letters (a – c) indicated significant differences among all treatment groups ($p < 0.05$).

demonstrated that MOS treatment had significant effects on the content of CORT [$F(1, 28) = 5.5$, $P = 0.0263$], and CRH [$F(1, 28) = 13.67$, $F(1, 28) = 13.67$]. An interaction effect between genotype and MOS treatment was also observed on the CORT content [$F(1, 28) = 6.29$, $P = 0.0182$]. As shown in Fig. 4**A**, the CORT level in AD mice was significantly higher than WT mice ($p < 0.05$), which was reversed by MOS treatment ($p < 0.01$). MOS also down-regulated the CRH level in AD mice (Fig. 4**B**) ($p < 0.01$). The expression of Nr3c1, Nr3c2, and Crhr1 in AD mice were significantly higher than the WT mice (Fig. 4**C-E**) ($p < 0.01$), whilst MOS treatment significantly down-regulated the mRNA expressions of these genes in AD mice (Fig. 4**C-E**) ($p < 0.01$). Besides, studies showed that the overactive HPA axis decreases the synthesis of norepinephrine (NE), thereby damaging neurons and emotional abnormalities (Oldehinkel and Bouma, 2011). Here, two-way ANOVA analysis indicated that a significant effect of MOS treatment [$F(1, 28) = 5.676$, $P = 0.0242$], and an interaction effect [$F(1, 28) = 7.518$, $P = 0.0105$] on the content of Serum NE. Post hoc analysis showed that the serum level of NE in AD mice was generally lower than WT mice (Fig. 4**F**) ($p < 0.05$), while MOS treatment significantly enhanced the NE level in the AD mice ($p < 0.01$). Overall, these results indicated that AD mice mitigated the HPA axis hyperactivity, whereas MOS could improve the endocrine function of the HPA and secretion of NE in AD mice.

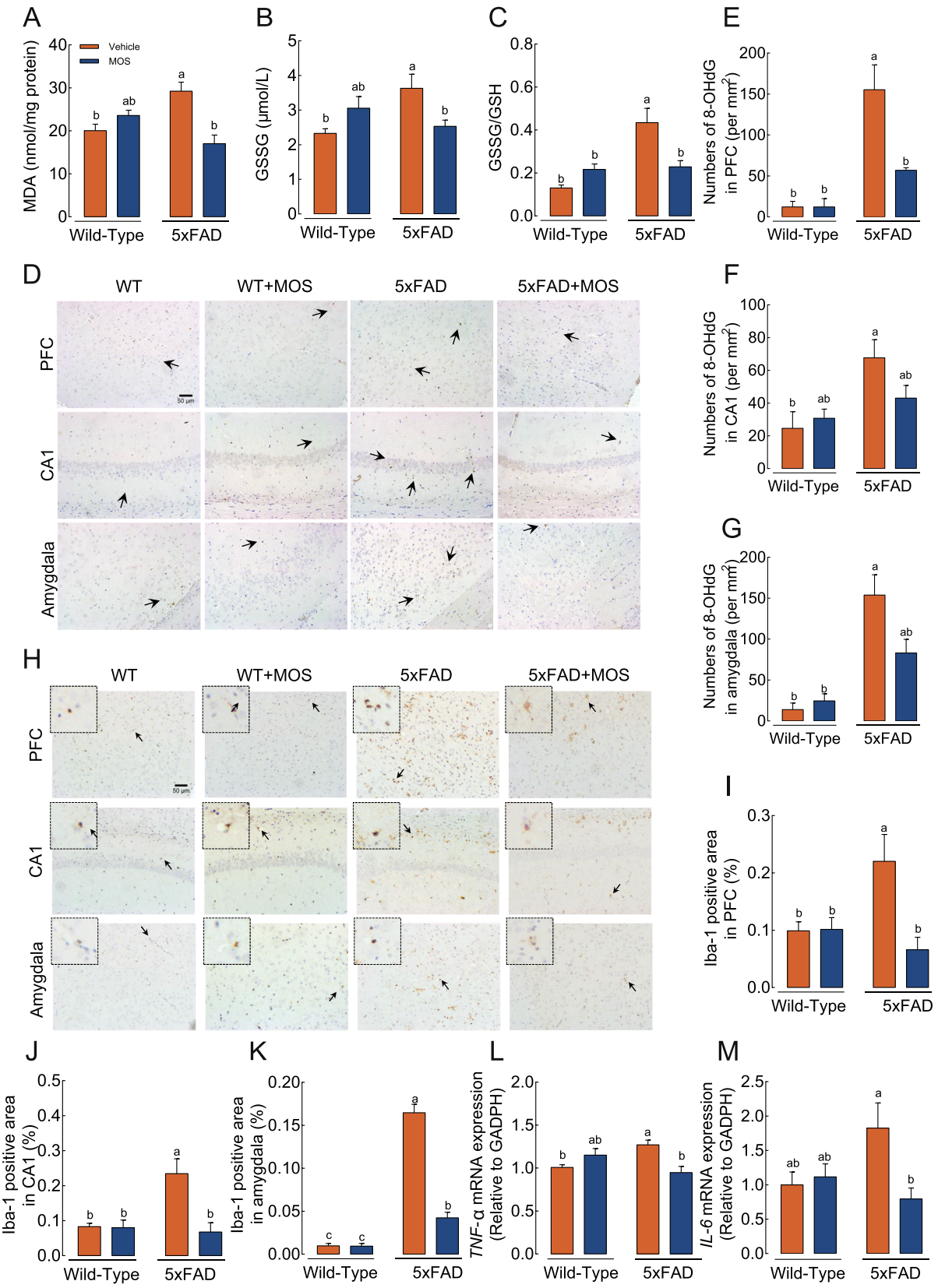

Fig. 3. Effects of MOS Treatment on Oxidative Stress and Inflammatory Responses of Brain in 5xFAD Mice. **(A-C)** The levels of MDA, GSSG, and the ratio GSSG/GSH in the brain (n = 8). **(D)** Representative images of immunohistochemistry (IHC) of 8-OHdG in the brain. **(E-G)** Quantification of 8-OHdG levels based on IHC-stained sections by ImageJ software (Representative images were captured from 6 slices of 3 mice per group). **(H)** Representative images of IHC staining of Iba-1 in the brain. **(I-K)** Quantification of Iba-1 positive area based on IHC-stained sections by ImageJ software (Representative images were captured from 6 slices of 3 mice per group). **(L-M)** The mRNA expression of TNF-α and IL-6 in the brain (n = 4). Data are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test. The letters (a – c) indicated significant differences among all treatment groups ($p < 0.05$).

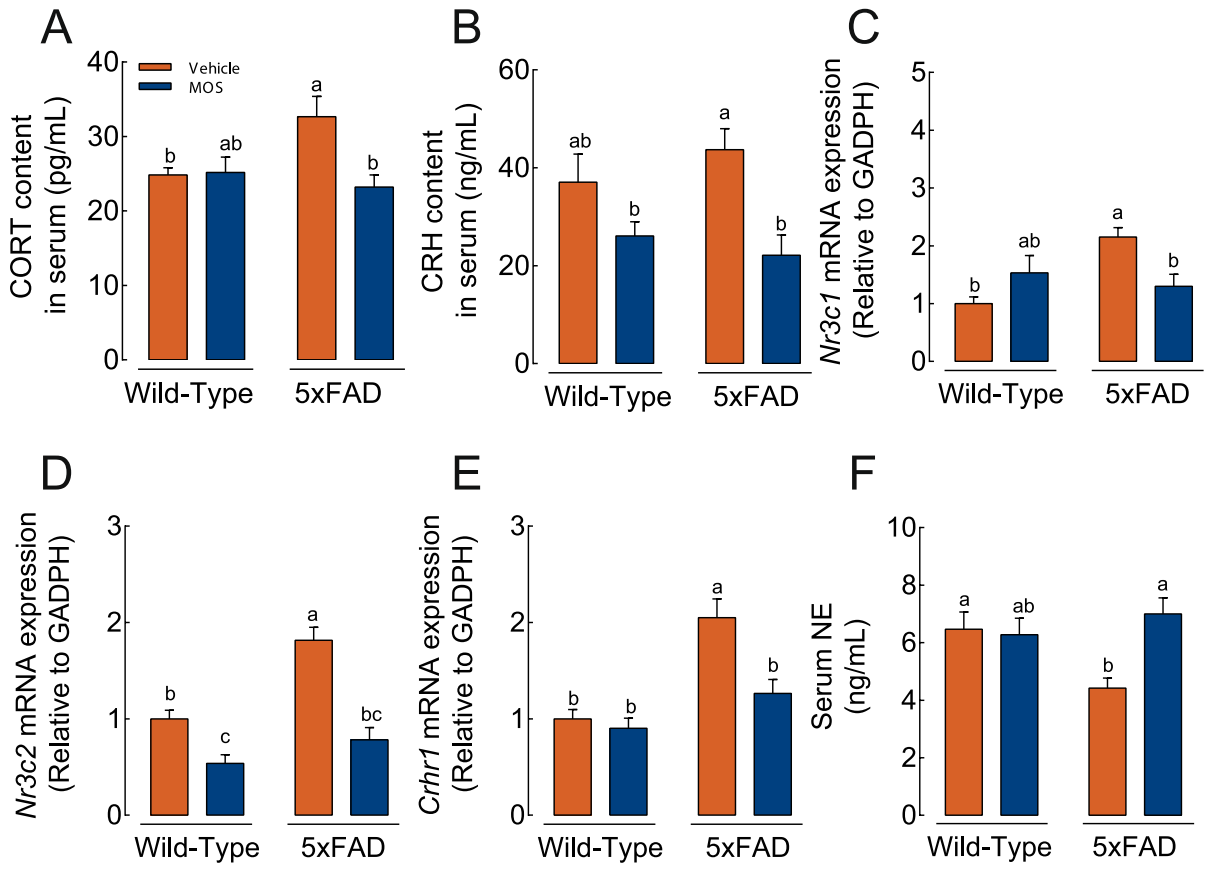

Fig. 4. Effects of MOS Treatment Balanced Hormonal Levels of HPA Axis and Monoamine Neurotransmitters Levels in 5xFAD Mice. (A-B) The levels of CORT and CRH in the serum (n = 8). **(C-E)** The mRNA expression of Nr3c1, Nr3c2, and Crhr1 in the hypothalamus (n = 4). **(F)** The levels of NE in the serum (n = 8). Data are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test. The letters (a − c) indicated significant differences among all treatment groups ($p < 0.05$).

3.6. Effects of MOS treatment on gut microbiome composition and SCFA formation in 5xFAD mice

The gut microbiome analysis was conducted by 16S rRNA sequencing in the mice feces. The results indicated that the α-diversity, including ACE index, Shannon index, and observed species were lower in AD mice than in WT mice (**Fig. S2A-C**). There were 23 and 17 specific OTUs in the WT group and AD group, respectively. Besides, there were 30 specific OTUs in the AD + MOS group (Fig. 5**A**). The partial least squares discrimination analysis (PLS-DA) plot illustrated a clear and separate clustering of the samples among different groups (Fig. 5**B**). A separation was observed between the diversity of the WT and AD mice. There was also a distinct separation between the diversity of the AD and AD + MOS groups. Moreover, there were 20 significantly different zOTUs between AD and AD + MOS groups, three of which belonged to *Ruminococcus* and one about *Lactococcus* (Fig. 5**C**). MOS treatment elevated the relative abundance of bacteria such as *Prevotella* and *Oscillospira* (Fig. 5**C**). Taxonomy-based analysis of samples from groups was further to determine the fecal microflora composition of AD mice was significantly different from that of MOS treated mice (**Fig. S2D**). At the genus level, the relative abundance of *Lactobacillus* was significantly elevated by MOS treatment in the AD mice (Fig. 5**D**). AD mice exhibited a higher relative abundance of *Helicobacter* than WT mice, while MOS treatment prominently reduced its level (Fig. 5**E**). These results indicated that the MOS significantly altered the gut microbiome in AD mice.

The content of SCFAs in feces and serum was determined (Fig. 5**F** and **G**). It was demonstrated that MOS treatment had significant effects on the content butyrate in faeces [F (1, 24) = 5.697, P = 0.0252], and serum [F (1, 24) = 7.62, P = 0.0109]. Post hoc analysis showed that MOS treatment significantly improved the butyrate levels but not propionate and valerate in both feces and serum in AD mice (Fig. 5**F-G** and **Fig. S2E-F**) ($p < 0.05$). Consistently, we also found that the abundance of butyrate-producing bacteria was significantly enhanced after MOS treatment. MOS significantly increased the abundance of *Clostridium_pasteurianum*, *Lachnospira*, *Phascolarctobacterium*, and Veillonellaceae (Fig. 5**H-K**). These results demonstrated that the MOS treatment upregulated SCFAs generation and related-microbes enrichment in AD mice gut.

3.7. Effects of MOS treatment on gut barrier integrity and hormones secretion of colon in 5xFAD mice

The H&E staining indicated that MOS significantly improved the exhibited severe pathological changes such as loss of goblet cells and shrunk crypts in the colon AD mice (Fig. 6**A** and **Fig. S3A-B**) ($p < 0.01$). Furthermore, the expression of claudin-1, a tight junction (TJ) protein, was detected in colon tissue by using immunohistochemical staining. As shown in Fig. 6**A and B**, supplementary MOS significantly improved the decrease of claudin-1 expression in AD mice ($p < 0.05$), which was consistent with the mRNA expressions of TJ proteins results, including ZO-1 and claudin-1 (Fig. 6**C** and **Fig. S3C**) ($p < 0.01$). Moreover, MOS treatment had a main effect on the serum LPS level [F (1, 28) = 12.2, P = 0.0016]. Besides, there was a significant interaction between genotype and MOS on LPS level [F (1, 28) = 12.46, P = 0.0015]. As shown in Fig. 6**D**, the serum LPS level in AD mice was significantly higher than the WT mice ($p < 0.01$), while MOS prevented the leak of LPS in the AD mice ($p < 0.01$). This result indicated that MOS protected the gut barrier integrity in the AD mice.

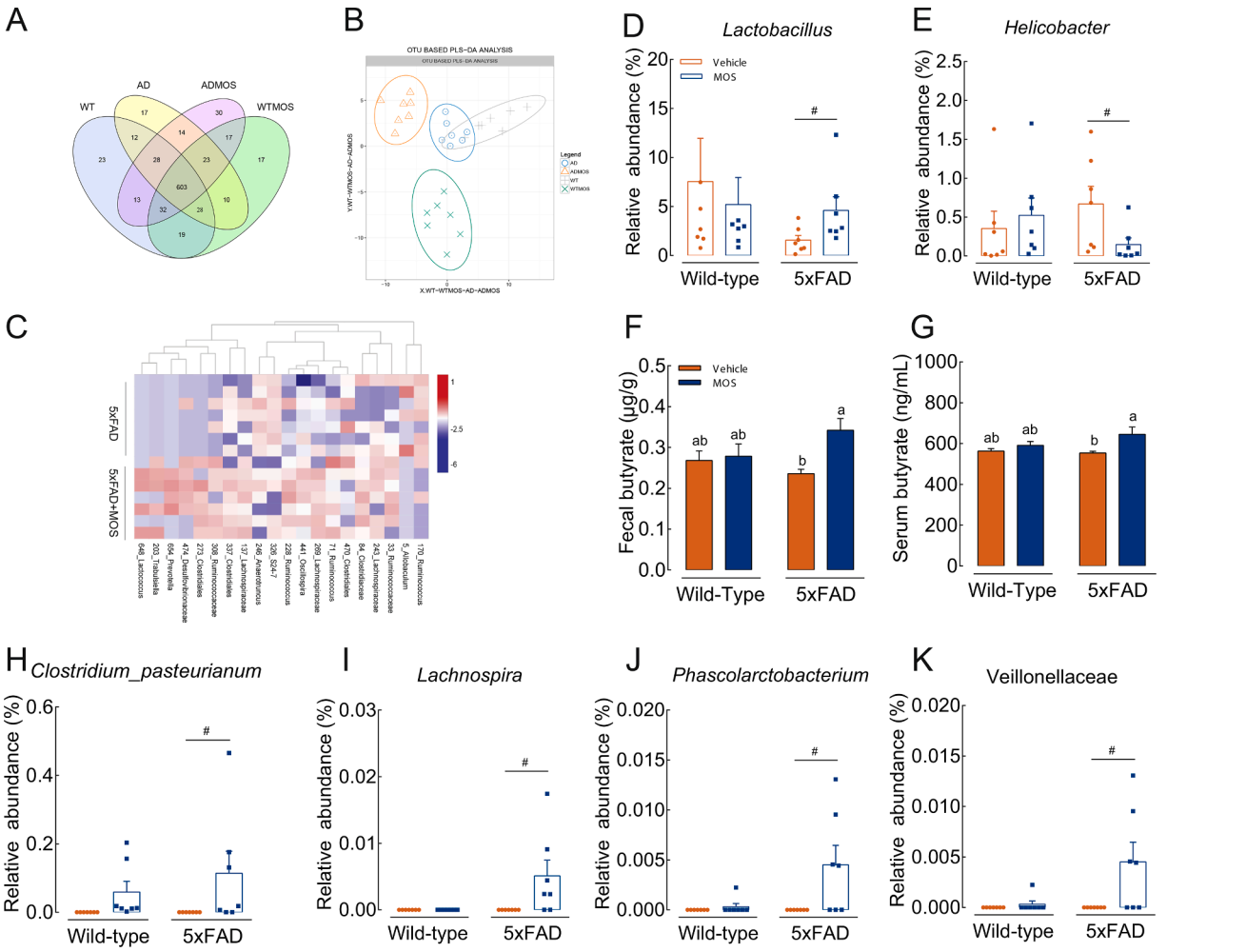

Fig. 5. Effects of MOS Treatment on Gut Microbiome Composition and Formation of SCFAs in 5xFAD Mice. (A) Venn diagrams illustrating the discrepancy of OTUs among the different treatment groups. **(B)** The result of Partial least squares discrimination analysis (PLS-DA) of each group. **(C)** Heatmap of the gut microbiota at the OTU level and the gradient of color was arranged from blue (low abundance) to red (high abundance). The relative abundance of **(D)** Lactobacillus and **(E)** Helicobacter. **(F)** Fecal levels of butyrate, and **(G)** Serum levels of butyrate. The relative abundance of **(H)** Clostridium_pasteurianum, **(I)** Phascolarctobacterium, **(J)** Lachnospira, and **(K)** Veillonellaceae. Microbiome sequencing was performed by data of WT group, WT + MOS group, AD group, AD + MOS group (n = 7). The data of gut microbiota are presented as mean ± SEM and statistical significance was determined by Wilcox test, n = 7, (*p < 0.05, **p < 0.01, compared with the WT group, #p < 0.05, ##p < 0.01, compared with the AD group). Data of the SCFAs levels are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test, n = 7. The letters (a – c) indicated significant differences among all treatment groups (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It has been reported that SCFAs could interact with the free fatty acid receptors (FFARs) and promote the secretion of gut hormones such as glucagon-like peptide 1 (GLP-1), thereby regulating learning, memory, and mood in the brain (Dalile et al., 2019). Here, the expression of FFAR2 and FFAR3 mRNA was found to be increased in the colon of MOS-treated AD mice (Fig. 6**E and F**) ($p < 0.01$). Consistently, the treatment of MOS significantly upregulated GLP-1 in AD mice in the colon tissue, compared with the vehicle group (Fig. 6**G**) ($p < 0.05$).

3.8. Correlation analysis between the Fecal/Serum butyrate levels and other biochemical index

To elucidate the correlation between the butyrate levels and these biochemical indexes, the Spearman correlation analysis was conducted (Fig. 6**H**). The serum butyrate levels, enriched by MOS treatment, exhibited significantly positive correlation with target time in Morris water maze test (r = 0.77), NE (r = 0.66), *Clostridium pasteurianum* (r = 0.78), and *Lactobacillus* (r = 0.6), but negatively correlated with CORT (r = -0.62), GSSG (r = -0.73), MDA (r = -0.71), which is consistence with the results found in feces butyrate. Moreover, we found that *Lactobacillus* was statistically related with Morris water maze test, including target time (r = 0.64). In addition, the butyrate-producing bacteria *Clostridium_pasteurianum* was positively associated with target time (r = 0.66) in the Morris water maze test but negatively correlated with marble buried (r = -0.72) and MDA in the cortex (r = -0.64). It has also been found that Veillonellaceae was negatively related with LPS (r = -0.71), CRH (r = -0.7), and GSSG/GSH (r = -0.71).

Besides, the multivariate regression analysis was also conducted to predict AD mice's cognitive performance based on the alteration of other indexes. As shown in **Table S2**, all predictors, including Butyrate_F, MBT_Marble_Buried, NE_S, CORT_S, MDA_c, g_*Lactobacillus*, and g_*Helicobacter*, explained 97.45% of the variance in the cognition abilities of the AD mice.

3.9. Effects of SCFAs treatment on behaviors and brain function in 5xFAD mice

The experimental timeline was as shown in Fig. 7**A**. The results of food intake, water intake, and body weight gain, suggesting that the SCFAs treatment had no effects on the physiological basis of mice

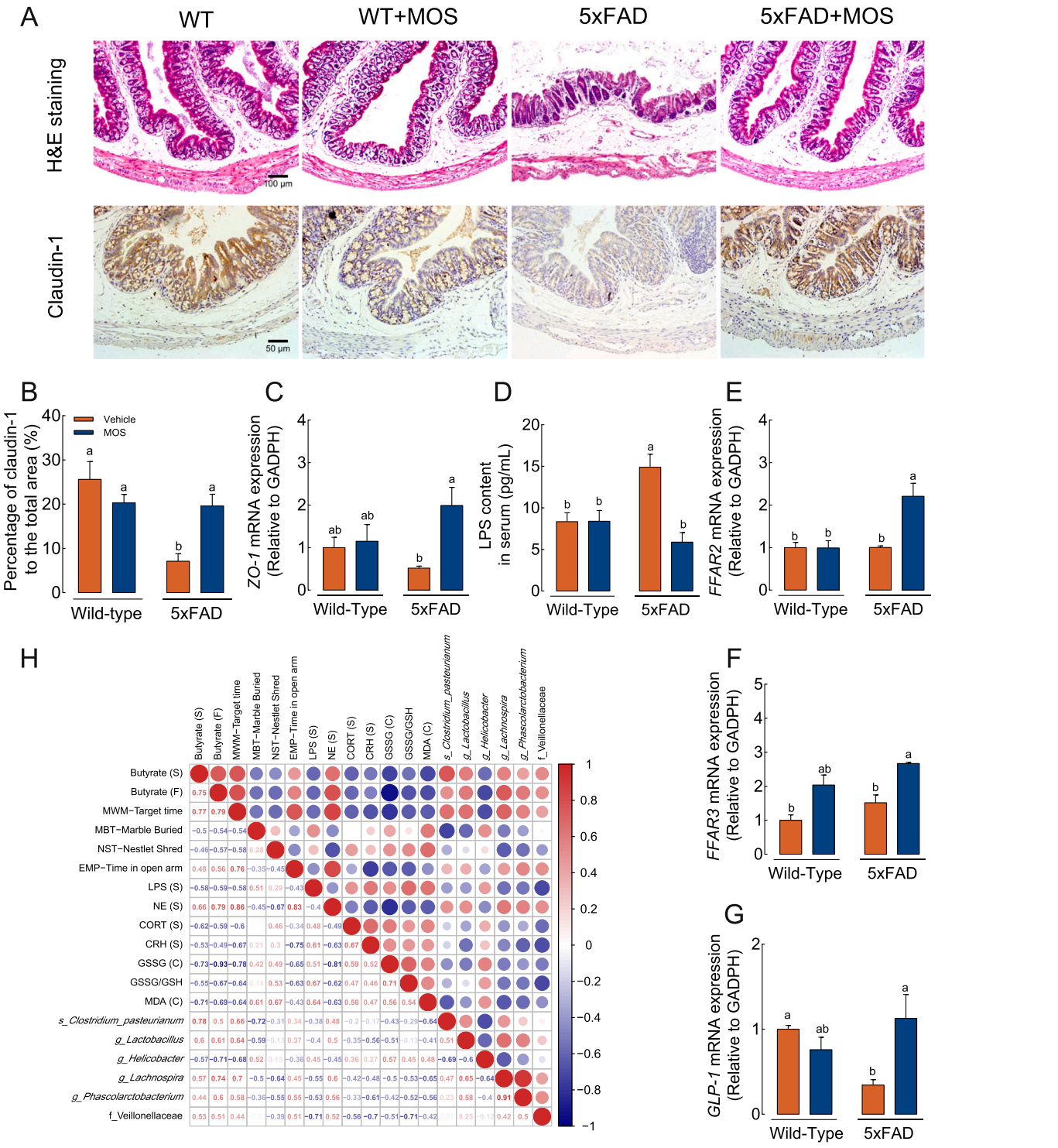

Fig. 6. Effects of MOS Treatment on Histopathological Changes and Hormones Secretion of Colon in 5xFAD Mice. (A) Representative images of H&E staining and IHC staining of claudin-1 in the colon for each group. **(B)** Quantitative immunohistochemical analysis of claudin-1 protein in the colon: the positive region was identified by ImageJ software, and the area ratio with the colon wall region was calculated (Representative images were captured from 6 slices of 3 mice per group). **(C)** The mRNA expression of ZO-1 in the colon for each group (n = 4). **(D)** The levels of LPS in the serum (n = 8). **(E-G)** The mRNA expression of FFAR2, FFAR3, and GLP-1 in the colon (n = 4 mice per group). **(H)** Correlation Analysis between the fecal/serum butyrate Levels and Other Biochemical Index. In the right of the matrix, the size and color of the circles indicate the degree of correlation. (red represents a positive correlation, and blue represents a negative correlation), and the corresponding value of the correlation index can be found on the right. The parameters for the cortex, feces, and serum were labeled as C, F, and S, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

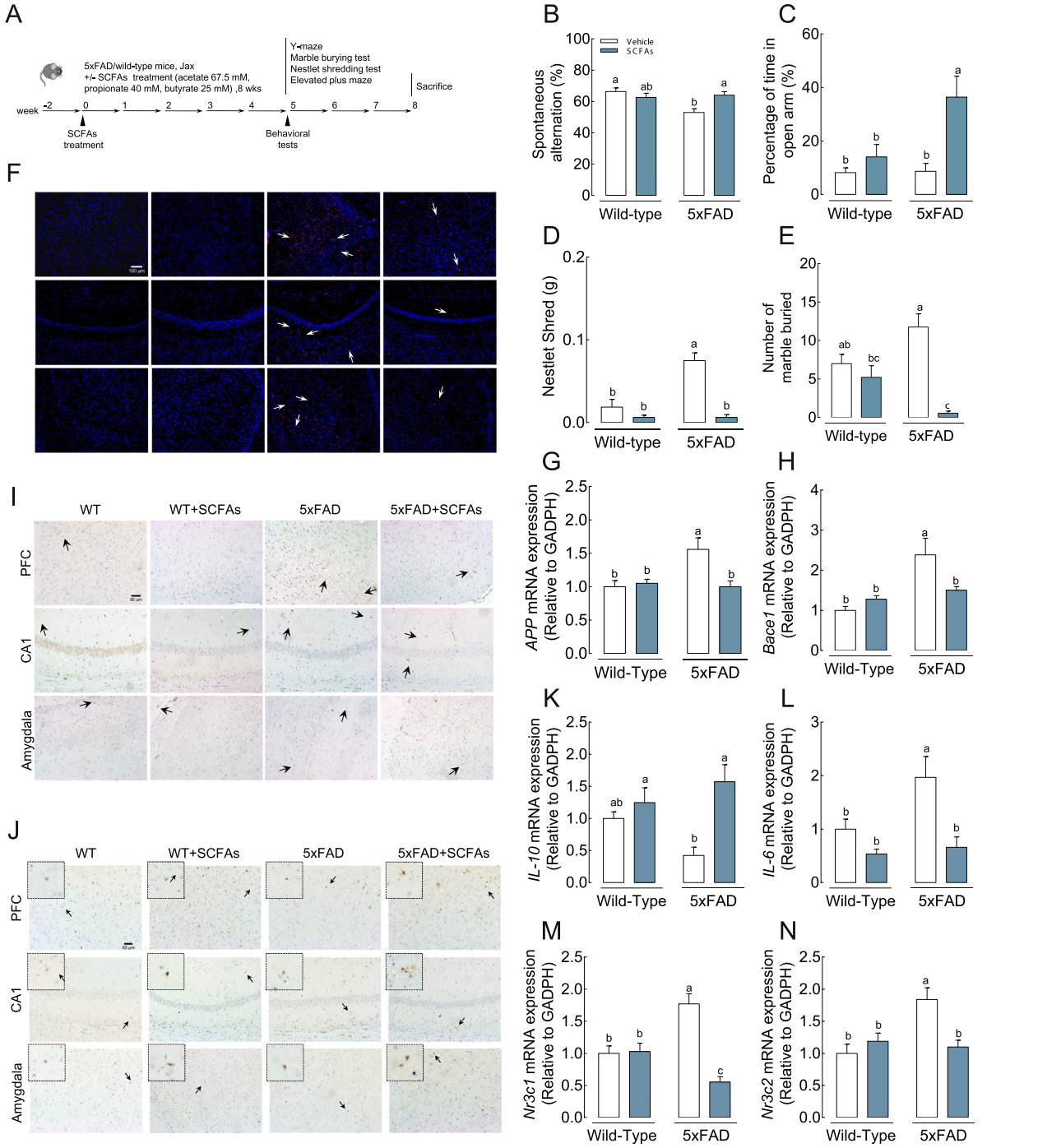

Fig. 7. Effects of SCFAs Treatment on Behaviors and Brain Function in 5xFAD Mice. **(A)** Experimental workflow of animal treatments (n = 8 mice per group). **(B)** Spontaneous alternation (%) in Y maze test, **(C)** percentage of time in open arm (%) in the EPM test, **(D)** nestlet Shred (g) in the NST, and **(E)** number of marble buried in the MBT. **(F)** Representative images of immunofluorescence staining of the brain. The mRNA expression of **(G)** APP and **(H)** Bace1 in the cortex (n = 4 mice per group). **(I)** Representative images of IHC staining of 8-OHdG in the brain. **(J)** Representative images of IHC staining of Iba-1 in the brain. **(K)** The mRNA expression of IL-10 and **(L)** IL-6 in the cortex (n = 4 mice per group). The mRNA expression of **(M)** Nr3c1 and **(N)** Nr3c2 in the hypothalamus (n = 4 mice per group). Data are presented as mean ± SEM, and statistical significance was determined by two-way ANOVA with Tukey's test. The letters (a – c) indicated significant differences among all treatment groups ($p < 0.05$).

(**Fig. S4A-D**). As shown in Fig. 7**B-E**, the significant effect of SCFAs treatment on spontaneous alternation of the Y maze test [$F(1, 28) = 9.516$, $P = 0.0046$], the time spent in open arms in EPM [$F(1, 28) = 12.04$, $P = 0.0017$], cotton shredding in NST [$F(1, 28) = 34.67$, $P < 0.0001$], and marble buried in MBT [$F(1, 28) = 24.91$, $P < 0.0001$] were observed. The result of the Y maze test indicated that SCFAs treatment significantly improved the working memory of AD mice. (Fig. 7**B**) ($P < 0.05$). The SCFAs group had more time spent in open arms in EPM (Fig. 7**C**) ($p < 0.01$), fewer nestlet shreds in NST (Fig. 7**D**) ($p < 0.01$), and fewer buried beads in MBT (Fig. 7**E**) ($p < 0.01$) than the vehicle group in AD mice ($p < 0.01$), which indicating SCFAs could alleviate the behavioral disorders of AD mice.

Moreover, H&E and immunofluorescence staining revealed that SCFAs alleviated neuron shrinks and reduced amyloid protein aggregation in the AD mice brain (**Fig. S4E,** Fig. 7**H, and S4F-H**). The mRNA expressions of APP and Bace1 were also significantly suppressed by the SCFAs treatment in the brain of AD mice (Fig. 7G-H and **S4I-J**) ($p < 0.01$). Two-way ANOVA analysis showed that SCFAs had a main effect on the levels of MDA [$F(1, 28) = 50.72$, $P < 0.0001$]. There was also a significant interaction between genotype × MOS on the levels of GSSG/GSH [$F(1, 28) = 29.68$, $P < 0.0001$]. An 8-week SCFAs intervention significantly reduced oxidative damages by decreasing the levels of MDA (**Fig. S5A**) ($p < 0.01$), the ratio of GSSG/GSH (**Fig. S5B**) ($p < 0.01$), and the 8-OHdG levels in the brain of AD mice (Fig. 7**I and S5C-E**) ($p < 0.01$). It has also been found that the SCFAs treatment effectively decreased the Iba-1 positive area and the release of inflammatory cytokines in the AD mice brain (Fig. 7**J,** **Fig. S5F-H,** and **7 K-L**) ($p < 0.01$).

Additionally, two-way ANOVA analysis indicated that the significant effect of SCFAs treatment on the CORT levels [$F(1, 28) = 50.72$, $P < 0.0001$], and NE levels [$F(1, 28) = 55.43$, $P < 0.0001$]. SCFAs significantly reduced the CORT levels (**Fig. S5I**) ($p < 0.01$) and elevated the NE levels (**Fig. S5J**) ($p < 0.01$) in the AD mice. Consistently, SCFAs also reduced the mRNA expressions of Nr3c1, Nr3c2, and Crhr1 in AD mice brain (Fig. 7**M-N** and **S5K**) ($p < 0.01$).

4. Discussion

The current study found that MOS supplementation significantly attenuates the cognitive deficits and anxiety-like behaviors in the male transgenic AD mice model. MOS treatment significantly reduced the Aβ accumulation and suppressed the expressions of APP and Bace1 in the brain of AD mice. Importantly, MOS treatment significantly inhibited the neuroinflammation and oxidative stress in the CNS of the AD mice, which partly explained the neuroprotective effects of MOS. MOS also balanced the HPA axis related hormone levels. Importantly, we found that MOS substantially reshaped the gut microbiome of AD mice and enhanced the formation of the microbial metabolites SCFAs. The correlation analysis indicated that the levels of SCFAs were highly correlated with behavioral changes and redox status. The SCFAs supplementation experiment also revealed that SCFAs significantly prevented cognitive impairment and anxiety-like behaviors, accompanied by reducing the neuroinflammation and oxidative stress in the AD mice brain.

MOS has been widely reported to have beneficial bioactivities, including antioxidant, anti-inflammatory, and gut-protective effects. Generally, MOS are mixtures of oligosaccharides formed by D-mannose linked through β-(1,4) linkages (Yui et al., 1992). The degree of polymerization of mannose residues varies from 2 to 10. It has been revealed that MOS could be fermented by *Lactobacillus* in the digestion system (Sharma et al., 2018). Our recent study indicated that 100–200 mg/kg/d supplementation of MOS significantly suppressed the lipid accumulation and appetite in diet-induced obesity mice via reshaping the gut microbiota structure and enhancing the SCFAs formation (Yan et al., 2019). It has been reported that MOS stimulated the abundance of some beneficial genera microbes and the generation of SCFAs in an *in vitro* experiment that was fermented by human feces (Pérez-Burillo et al., 2019). Previous studies indicated that 1%-5% (*w/w*, in diet) MOS treatment significantly altered the gut microbiota composition and improved the SCFAs formation in rat models (Asano et al., 2004; Hoving et al., 2018a). Moreover, a double-blind, placebo-controlled human study demonstrated that MOS intake (3 or 5 g/day for three weeks) also significantly impacted healthy individuals' gut microbiome (Walton et al., 2010). In the current study, the dose of MOS is 0.12% (*w/v*) in drinking water, which is approximately 200 mg/kg/d for the AD mice. The equivalent dose for humans is 21.99 mg/kg/day, based on the Meeh-Rubner equation (Ohwada, 1992).

Our previous study indicated that MOS upregulated the relative abundance of *Lactobacillus* in the obese mice, positively associated with the increase of SCFAs and negatively associated with the appetite changes (Yan et al., 2019). Consistently, we found that MOS treatment significantly improved the level of *Lactobacillus* in AD mice (Fig. 5). Supplementation of *Lactobacillus fermentum* strain NS9 has been demonstrated to improve the cognitive function and hippocampal mineralocorticoid receptor and N-methyl-D-aspartate receptors' expressions in an ampicillin-treated rat model (Wang et al., 2015). Similarly, the current study also indicated that the relative abundance of *Lactobacillus* is also positively associated with the cognitive performance in the Morris water maze test (Fig. 6**H,** $r = 0.64$). Moreover, the current study indicated that the level of *Helicobacter* in the AD mice gut was higher than in the wild-type mice (Fig. 5). However, MOS supplementation significantly reduced the level of *Helicobacter*, which is also negatively correlated with the improvement of cognitive function (Fig. 6**H,** $r = -0.68$). A previous study indicated that nearly 89.9% of the mild cognitive disorder patients are infected with the *Helicobacter* (Kountouras et al., 2007). It has been found that intraperitoneal injection of *Helicobacter pylori* significantly increased the Aβ accumulation in the cortex and hippocampus in a rat model, which is possibly related to the elevated activity of Bace1 (Wang et al., 2014). These results demonstrated that the alteration of gut microbiota composition is highly related to the cognitive function changes in AD mice.

The microbial metabolites generated in the gut have crucial impacts on host health (Kasubuchi et al., 2015). Here in the present research, it has been found that the endotoxin, LPS, generated from Gram-negative bacteria, was significantly elevated in the serum of the AD mice, compared with the wild-type mice, which is highly correlated to the leak of the gut barrier (Fig. 6). However, the expressions of TJ protein claudin-1 were significantly improved in the MOS-treated mice, which were accompanied by the decrease of LPS leak into the serum. The leak of LPS stimulates neuroinflammation in the CNS (Catorce and Gevorkian, 2016). In the present study, the overactivation of microglia in the cortex, hippocampus, and amygdala was significantly suppressed by the MOS treatment, accompanied by the decreasing expressions of inflammatory cytokines. The neuroinflammatory responses are also related to the redox status in AD progress (Agostinho et al., 2010). The MOS supplementation significantly inhibited the oxidative stress in the AD mice brain. These results provided a clue that MOS maintains the gut barrier integrity and suppresses the leaking of the toxic metabolites and subsequent neuroinflammation and oxidative stress, which partly explain how MOS could improve the cognitive function in the AD mice.

SCFAs are the major metabolites generated from complicated carbohydrates fermentation in the gut (Holscher, 2017). It has been found that SCFAs have beneficial effects on maintaining gut barrier integrity by enhancing the expression of TJ proteins and protecting intestinal mucosa (Parada Venegas et al., 2019; Silva et al., 2018). Moreover, numerous studies demonstrated that the neuroprotective effects of some specific oligosaccharides are related to the SCFAs formation (Sun et al., 2019; Wang et al., 2019a; Xin et al., 2018). It has also been reported that SCFAs could provide its interaction with the receptors such as FFAR2/3 expressed in the gut and subsequently enhancing the generation of GLP-1, which will further influence the emotion, cognition, and pathophysiology of mental disorders via the circulating system and vagus nerves (Dalile et al., 2019). Some *in vitro* studies revealed that SCFAs interface with $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (Ann Dipika Binosha Fernando et al., 2014; Ho et al., 2018). A recent study indicated that injection of butyrate significantly reduced the Aβ accumulation in the 5xFAD mice brain (Fernando et al., 2020). Here, in our research, it has been found that MOS supplementation also enhanced the butyrate formation, which is positively correlated to the improvement of cognitive function (Fig. 6**H,** $r = 0.77$). MOS improved the levels of SCFAs formation-related microbiota, such as *Clostridium_pasteurianum*, *Lachnospira*, *Phascolarctobacterium*, and Veillonellaceae (Tsukahara et al., 2002; Vacca et al., 2020; Vital et al., 2017; Wu et al., 2017). The correlation analysis also confirmed that these probiotics' relative abundance was positively correlated with the serum and fecal levels of butyrate (Fig. 6**H**). Moreover, the present research

demonstrated that supplementation of SCFAs also significantly alleviated the cognitive decline and Aβ accumulation in the AD mice. SCFAs treatment also suppressed the inflammatory responses and oxidative stress in the CNS of AD mice (Fig. 7). These results indicated that SCFAs play essential roles in mediating the neuroprotective effects of MOS in the AD mice.

Furthermore, the present research demonstrated that MOS treatment alleviated mental disorders by decreasing the obsessive-behaviors and anxiety-like behaviors in the AD mice. HPA axis plays a pivotal role in regulating mental health (Kallen et al., 2008). The elevated glucocorticoid (GC) level, which triggers GC receptors (GR) signaling damaged, has been proved to be a direct element for HPA axis hyperactivity (Canet et al., 2019). Studies in human and mice have shown that high concentrations of GCs, simply penetrated the brain, which induced pro-inflammatory and neurotoxic cytokines in several brain regions (Munhoz et al., 2010; Duque Ede and Munhoz, 2016), hippocampal neuronal damage (Anacker et al., 2013), and oxidative injury (Sato et al., 2010). Thus, dysfunction of GR and mineralocorticoid receptor expression can occur in specific pathological, emotional, and cognitive conditions (Keller et al., 2017). Of note, previous evidence also showed that both CRH and GCs could exacerbate AD pathology in the brains of transgenic mouse models (Futch et al., 2017). The overactive HPA axis, which could reduce the synthesis of central monoamine neurotransmitters, including 5-HT and NE, thereby damaging hippocampal neurons and eventually leading to emotional abnormalities (Oldehinkel and Bouma, 2011; Pariante and Lightman, 2008). Here, it has been found that MOS treatment significantly down-regulated the serum levels of CORT and CRH, upregulated the circulating NE level, and normalized the gene expressions of Nr3c1, Nr3c2, and Crhr1 in the hypothalamus. Interestingly, the enhanced butyrate level in the AD mice was negatively correlated to the levels of CORT (Fig. 6**H**, r = -0.62). A previous study consistently indicated that SCFAs treatment alleviated the stress-induced HPA axis dysfunction (van de Wouw et al., 2018). SCFAs could improve the NE release by stimulating FFAR3 in the primary-cultured mouse sympathetic cervical ganglion neurons (Inoue et al., 2012; Kimura et al., 2011). Consistently, the present study also demonstrated that SCFAs administration has similar effects on regulating the HPA axis disorders in the AD mice (Fig. 7). These results revealed that MOS could alleviate the mental disorders in the AD mice, which could be partly explained by enhancing the formation of SCFAs in the gut. However, future study is needed to investigate how SCFAs activate the NE formation and balance the HPA axis in the glucocorticoid receptor and SCFAs receptor knockout animal models. Moreover, in view of the significant sex difference in AD, the purpose and results of this study were only to explore the intervention effect of MOS on male AD mice, which may be generalizable only to males. Future study is needed to uncover the sex difference of the beneficial effects of MOS in AD progress.

In conclusion, the present study found that supplementation of MOS significantly attenuates the cognitive and mental deficits in the 5xFAD mice, which could be partly explained by the reshaped microbiome and enhanced SCFAs formation in the gut. The gut microbiota-SCFAs-brain axis plays a crucial role in suppressing neuroinflammation, preventing oxidative stress in the CNS, and balancing the HPA axis, which is possibly the fundamental mechanism of the neuroprotection of MOS treatment. The beneficial effects of MOS on changes in microbiota/metabolites/genes should be further evaluated in clinical trials. Once validated, MOS, as a prebiotic, can be translated into a novel microbiota-targeted approach for managing metabolic and neurodegenerative diseases.

Author contribution

QL and ZL designed the experiments; QL, YX, QW, JL, PL, XM, KL, and ZL performed and analyzed the experiments; QL, QW, and ZL interpreted the data; QL and WC prepared figures; QL and ZL wrote the manuscript; XL and ZL supervised the work. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2021.04.005.

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