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Research Article

Liquid-State Fermented *Ganoderma lucidum* GANO99 Regulates Gut Microbiota and Concomitantly Modulates the Behavioral Deficits and Neurohistopathological Hallmarks of Alzheimer's Disease in a Preclinical Transgenic Mouse Model of Alzheimer's Disease

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Amyloid beta (Aβ) is a neuropathological hallmark of Alzheimer's disease (AD). Oxidative stress promotes intracellular accumulation of Aβ in patients with AD. The effects of liquid-state fermentation products of *Ganoderma lucidum* without mycelia on AD improvement or prevention have not been reported. Therefore, this study aimed to determine whether the liquid-state fermented products of *G. lucidum* GANO99 can alter AD pathology in the brains of $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ model mice. Our results suggest that these products promote the expression of superoxide dismutase and inhibit the expression of Aβ and glial fibrillary acidic protein, thereby affecting behavioral, learning, and memory disorders in AD mice. In contrast, *G. lucidum* GANO99 may regulate the gut microbiota to improve AD pathology. Furthermore, these liquid-state fermented products increased the abundance of intestinal probiotics *Bifidobacterium* in AD mice. Clinical evidence suggests that these lactic acid bacteria can alleviate AD. Overall, the liquid-state fermented products of *G. lucidum* GANO99 can act as symbionts by reducing the fermentative role of intestinal microbes and chemo-heterotrophy in AD mice.

1. Introduction

Alzheimer's disease (AD) is an irreversible, progressive, and neurodegenerative condition that leads to the death of cranial nerve cells. It is characterized by symptoms such as the degeneration of neurons controlling thinking, memory, and language areas in the brain and the loss of linguistic competence, spatial memory, and recognition functions [1]. Pathological diagnosis of AD includes the presence of encephalopathy, senile plaques, and neurofibrillary tangles in the cerebral cortices. Despite the indeterminate pathogenesis of AD, some researchers propose that β-amyloid (Aβ) peptides and amyloid precursor proteins (APPs) are the pathogenic factors. These factors react with enzymes, such as BACE1 and γ-secretase, during proteolysis [2, 3].

A newly introduced AD mouse model, $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mouse, carries Swedish, Iberian, and Arctic mutations, which promote Aβ accumulation, resulting in a higher Aβ42/Aβ40 ratio, increased oligomerization, and decreased proteolysis triggering Aβ accumulation [4]. This novel model addresses the issues associated with APP overexpression. $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice exhibited memory impairment in the Y-maze test at

6 months and demonstrated enhanced compulsive behavior, reduced attention, and changes in anxiety-related test results in previous studies [5, 6].

Herein, we discuss using healthy foods, medications, or Chinese herbal medicines for treating AD. The hot water extract of *Ganoderma lucidum* possesses antioxidant, hypocholesterolemic, and memory effects [7–9]. Compared with *G. lucidum* fruiting bodies used in traditional Chinese herbal medicine, liquid-state fermentation products of *G. lucidum* generated using the submerged fermentation technique are extracted from other parts of *G. lucidum*. These products have different properties, such as form, chemically active ingredients, and pharmacological action. The components of *G. lucidum* mycelium-fermented liquid have antitumor activity [10] and hepatoprotective properties [11], and it influences gut microbiota and cardiovascular diseases [12]. However, the effects of liquid-state fermentation products of *G. lucidum* without mycelia on AD improvement or prevention have not been reported.

Recent studies have demonstrated the important role of gut microbiota in AD pathogenesis [13]. The gut-brain axis is highly interconnected between the central nervous system and enteric nerves. The intestinal microbiota indirectly affects the hypothalamic-pituitary-adrenal system and activates neurons that affect the central nervous system [14]. Loss of cognitive function and memory may impair the function of the blood-brain barrier and induce abnormal central nervous system function due to the destruction or absence of microbiota [15]. Moreover, administering probiotics to transgenic mice can reduce amyloid accumulation and neuroinflammation, indicating that improving the gut microbiota can alleviate AD pathogenesis [1, 16]. Therefore, AD may be related to gut microbiota imbalance. The gut microbiota depends mainly on polysaccharides, which are bioactive components of *G. lucidum*. Thus, oral Chinese herbal medicines can treat central nervous system-related pathologies. Extracts from *G. lucidum* fruiting bodies or spores have been used to improve AD. Thus, in this study, we aimed to determine whether liquid-state fermented products of *G. lucidum* (GANO99 strain) can improve AD pathogenesis and gut microbiota.

2. Materials and Methods

2.1. Sample Preparation. The *G. lucidum* GANO99 strains were used from the shake culture in a liquid medium to produce liquid-state fermentation products. *G. lucidum* GANO99 solids were separated from the liquid-state fermentation products to remove mycelia and obtain a liquid-state fermentation broth. Syngen Biotech Co. Ltd. (Tainan, Taiwan) provided the *G. lucidum* GANO99 strains. The liquid culture filtrate was freeze-dried and then ground into fine powder for use in animal experiments. The *G. lucidum* GANO99 strain was deposited as DSM 33211 at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). *G. lucidum* GANO99 was maintained on potato dextrose agar plates and then cultured at 20~28°C for 7 days. Subsequently, the submerged fermentation was conducted using the medium comprising glucose (4~10%), soy peptone (0.5~2%), inorganic salts (0.01~0.3%), and minerals (0.01~0.3%) in a fermenter for 8 to 12 days under stirring and aeration conditions to obtain *G. lucidum* GANO99. The fermentation products were freeze-dried and stored at −80°C.

2.2. Animals. Adult male C57BL/B6-APP$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice carrying Arctic, Swedish, and Beyreuther/Iberian mutations were obtained from Dr. Takaomi C. Saido, Laboratory for Proteolytic Neuroscience, RIKEN Center for Brain Science, Japan. These mice had an APP construct containing a humanized Aβ region [4]. In addition, the C57BL/6J wild type (WT) without gene knock-in was used as blank control. APP$^{NL\text{-}G\text{-}F}$/$^{NL\text{-}G\text{-}F}$ transgenic mice model is introduced by APP overexpression using a knock-in approach. The mice display age-associated cognitive dysfunction starting at six-months old, including memory impairment [17]. The National Taiwan University Ethics Committee approved the experimental design and animal care (IACUC: NTU-104-EL-00073). The mice were bred in a small chamber with a constant temperature (21–22°C) and a 12 h dark-light cycle for lights-on time between 18:00 and 6:00.

The liquid-state fermented products of *G. lucidum* (GANO99) were dissolved in sterile water for mice oral administration. The mice (9 months old) were randomly classified into four groups ($n = 6$): (1) WT mice were administered sterile water as blank group; (2) AD mice (C57BL/B6-APP$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice) were administered sterile water for control group; (3) low-dose group: AD mice orally administered liquid-state fermented products of *G. lucidum* (GANO99) at 1X dosage (205 mg/kg BW); and (4) high-dose group: AD mice orally administered liquid-state fermented products of *G. lucidum* (GANO99) at 5X dosage (1,025 mg/kg BW). The mice were fed the samples via oral gavage once per day (5 days/week) for 4 months, and the behavioral tasks were evaluated. Brain tissues of euthanized mice were subsequently collected for histopathology analysis. All mice in the experiment were sacrificed, and their brain tissue was used for the immunohistochemistry (IHC) assay.

2.3. Behavioral Tasks

2.3.1. Y Maze. The setup consisted of three arms of equal length and angle. The mice were placed in one arm of the maze (start arm) and allowed to explore the maze with one arm closed for 5 min (training trial). After a 1 h intertrial interval, the mice were returned to the Y maze and placed at the start arm. Subsequently, the mice were allowed to freely explore all three arms of the maze for 10 min (test trial). The number of entries into and time spent in each arm, and the first choice of entry, were registered from video recordings by an observer blind to the genotype of the mice [18].

2.3.2. Morris Water Maze (MWM) Test. A circular pool (height: 40 cm, diameter: 120 cm) was filled with water, dyed black using dissolving food coloring, and maintained at 22–25°C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 0.5–1.0 cm below the water surface

in the northeastern quadrant of the pool. During the training trials, the mice were placed in the pool and allowed to remain on the platform for 20 s. They were returned to their cage during the second-trial interval. The mice that did not locate the platform within 60 s were placed on the platform for 20 s at the end of the trial. The mice were allowed to swim until they found the escape platform. Four such daily training trials (intertrial interval: 15–30 min) were conducted on 7 subsequent days. Data were averaged per trial day. Starting positions in the pool varied between four fixed positions, ensuring that each position was used on every training day [19].

The trial was completed once the mouse located the platform or 60 s elapsed. If the mouse failed to locate the platform in a given trial, the mouse was guided onto the platform and allowed to stay for 20 s. The latency to reach the platform was analyzed to confirm the learning behavior of the mouse. A single probe trial was conducted on day 8 to assess the integrity and strength of spatial memory 24 h after the last trial of the acquisition phase. The data in the probe trial were analyzed based on the time spent by the mice in the target quadrant and average proximity to the escape annulus. Escape latency, escape distance, time spent in the target quadrant, and swimming speed of each mouse were monitored.

2.3.3. Open Field Test (OFT). Each mouse was individually placed in the center of an open-field apparatus (40 cm × 40 cm × 40 cm) and allowed to explore freely for 10 min. The total distances traveled by each mouse were analyzed.

2.3.4. Elevated Plus Maze Test (EPMT). The elevated plus maze comprised two open arms (40 L × 10 W·cm in size) and two closed arms enclosed by white acrylic walls (20 cm high). The open and closed arms cross each other at a right angle and are located 50 cm above the floor. All arms were made of white acrylic plates radiating from a central platform (10 × 10 cm) to form a "+" sign. Each mouse was placed in the central platform facing one of the closed arms. The motion trail and time spent in the apparatus were recorded for 10 min.

2.4. Immunohistochemistry. The right hemispheres of the mouse brains were embedded in paraffin. The sections were incubated in Tris-Buffered saline (PBS) containing 0.1% Triton X-100 for 30 min and blocked with goat serum for 30 min at room temperature. After washing with PBS, the sections were incubated with primary antibodies against mouse monoclonal glial fibrillary acidic protein (GFAP; 1 : 250, sc-33673, Santa Cruz Biotechnology), anti-Aβ antibody (1 : 500, 800701, BioLegend), or anti-superoxide dismutase (SOD)-1 (1 : 500, GeneTex, GTX100554) at 4°C overnight. After washing with PBS, the sections were incubated with secondary antibodies (K500711, Dako REAL™ EnVision™ Detection System) for 20 min and then incubated with horseradish peroxidase-(HRP-) streptavidin reagent for 20 min. Finally, immunoreactivity was detected using 3,3-diaminobenzidine (DAB), followed by restaining with hematoxylin. The sections were mounted on microscope slides, dehydrated, and covered with coverslips. Finally, whole slides were imaged using an Olympus BX51 microscope (C&S, Japan). We analyzed 3 brain sections for immunostaining markers for each mouse. The images were analyzed using ImageJ software.

2.5. Gut Microbiota Analysis. Feces in the colon of each mouse were collected and immediately soaked in liquid nitrogen and stored at −80°C while mice were sacrificed. For 16S rRNA gene sequencing, the V3–V4 regions were amplified using a specific primer set (319F: 5′-CCTACGGGNGGCWGCAG-3′, 806R: 5′-GACTACHVGGGTATCTAATCC-3′) using the 16S Metagenomic Sequencing Library Preparation procedure (Illumina). In brief, 12.5 ng of gDNA was used for the PCR performed with KAPA HiFi HotStart ReadyMix (Roche) under the following condition: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min; and held at 4°C. The PCR products were monitored on 1.5% agarose gel. Samples with bright main strips around 500 bp were chosen and purified using the AMPure XP beads for library preparation.

The sequencing library was prepared using the 16S Metagenomic Sequencing Library Preparation procedure (Illumina). In brief, a secondary PCR was performed using the 16S rRNA V3–V4 region PCR amplicon and Nextera XT Index Kit with dual indices and Illumina sequencing adapters (Illumina). The indexed PCR product quality was assessed on the Qubit 4.0 Fluorometer (Thermo Fisher Scientific) and Qsep100TM system. An equal amount of the indexed PCR product was mixed to generate the sequencing library. Lastly, the library was sequenced on an Illumina MiSeq platform, producing paired 300 bp reads.

2.6. Statistical Analysis. The data are presented as mean ± standard error of the mean (SEM) ($n = 6$). Data were analyzed using one-way ANOVA and Duncan's *post hoc* test. Statistical significance was set at a p value of 0.05 or less. For microbiota analysis, pairwise ANOSIM (analysis of similarities) with permutations was conducted and evaluated using principal coordinate analyses (PCoA) based on different distance matrices, where p values were reported after Benjamini–Hochberg multiple testing correction. The value corresponding to the heatmap represents the Z score obtained by the abundance of each species in all groups. The Z score of a sample on a certain classification is the value of the average abundance of the sample on the category and all samples in the classification. LEfSe (linear discriminant analysis (LDA) effect size) uses nonparametric factorial Kruskal–Wallis (KW) sum-rank test to find species with significant differences in abundance and uses LDA to estimate the impact of abundance of each species to find out the communities or species that have a significant difference in the sample division. LEfSe analysis was employed for the screening value of LDA.

3. Results

3.1. Liquid-State Fermented Products of G. lucidum GANO99 Enables Ameliorating Memory Impairments and Anxiety-Related Behaviors. Figure 1(a) displays the

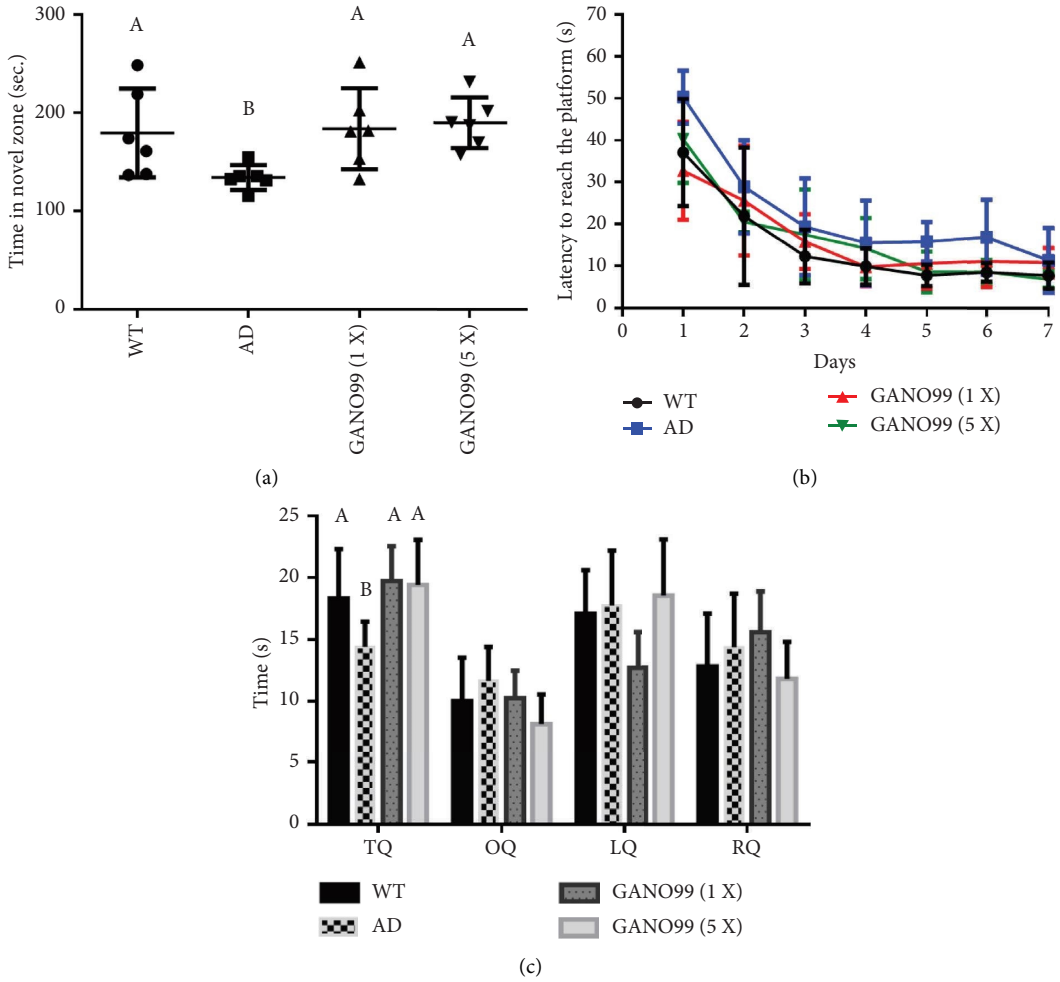


Figure 1: Evaluation of behavioral tasks included (a) Y maze, (b) Morris water maze (MWM), and (c) MWM-probe test in $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice administered with liquid-state fermented products of *G. lucidum* GANO99. All data are expressed as mean ± SEM ($n = 6$). *Significant difference was determined by one-way ANOVA ($p < 0.05$).

Y-maze test; we discovered that the learning and memory abilities of AD mice improved following treatment with liquid-state fermented products of *G. lucidum* GANO99. In the Morris water maze (MWM), treatment with the liquid-state fermented products of *G. lucidum* GANO99 alleviated memory impairment in AD mice (Figure 1(b)). In the MWM probe test, AD mice exhibited significantly shorter time and distance in the target quadrant than did WT mice. AD mice supplemented with the liquid-state fermented products of *G. lucidum* GANO99 exhibited significant alleviation of memory impairment (Figure 1(c)).

The learning and memory abilities of the mice were analyzed using behavioral tests. We compared the locomotor activity and anxiety-related exploration between WT and AD mice using the OFT and EPMT. Figure 2(a) shows the total distance traveled by the mice in the OFT, and no significant differences were observed between the groups. EPMT was used to evaluate anxiety-related behaviors, where increased or decreased exploration of the open arms indicated anxiolytic or anxiogenic behavior, respectively. Anxiety-related behavior of the mice was evaluated by comparing the residence time and distance traveled in the open and closed arms. We observed the time spent in the open arms by AD mice. *G. lucidum* GANO99 treatment markedly ameliorated the increased time spent in the open arms during the EPMT task (Figures 2(b)).

3.2. Liquid-State Fermented Products of G. lucidum GANO99 Elevate the Accumulation of Antioxidase and Reduce That of GFAP and Aβ Peptides in the Brain. Figure 3 illustrates the SOD activity in the cortex and hippocampus of AD mice. AD mice had significantly lower SOD expression in the hippocampus and cortex than did WT mice. However, treatment with the liquid-state fermented products of *G. lucidum* GANO99 increased the SOD level.

Excessive activation of GFAP, a common protein in the cerebrum, correlates with AD. Figure 4 illustrates the GFAP levels in mice treated with the liquid-state fermented products of *G. lucidum* GANO99 measured via IHC staining. AD mice had significantly higher GFAP expression in the hippocampus and cortex than did WT mice. However, GFAP levels were attenuated in AD mice by treatment with liquid-state fermented products of *G. lucidum* GANO99.

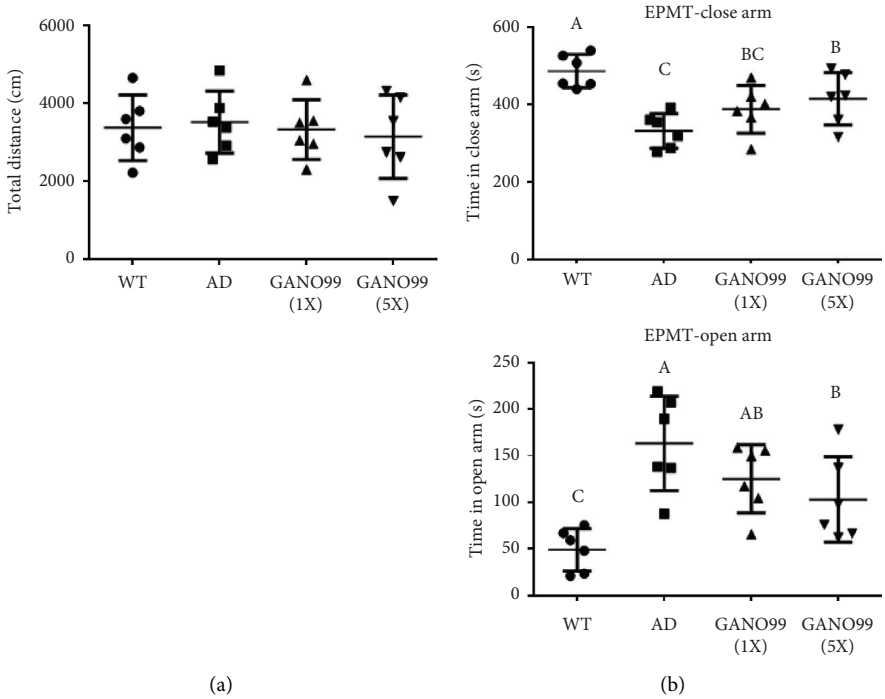


Figure 2: Evaluation of behavioral tasks included (a) OFT and (b) EPMT in $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice administered with liquid-state fermented products of *G. lucidum* GANO99. All data are expressed as mean ± SEM ($n = 6$). *Significant difference was determined by one-way ANOVA ($p < 0.05$).

Furthermore, Figure 5 indicates that Aβ accumulation was reduced in AD mice through supplementation with the liquid-state fermented products of *G. lucidum* GANO99.

3.3. Liquid-State Fermented Products of G. lucidum GANO99 Altered Gut Microbiome Composition. To explore whether the action of *G. lucidum* GANO99 against AD is mediated by the regulation of intestinal microbes, the microbiota composition and diversity in the feces of AD mice treated with liquid-state fermented products of *G. lucidum* GANO99 were evaluated. A Venn diagram was used to display the number and species of various groups. The analysis revealed 321 different types of bacteria in all groups. However, 11, 15, 7, and 22 operational taxonomic units (OTUs) were observed in WT, AD, AD + liquid-state fermented products of *G. lucidum* GANO99 (1x), and AD + liquid-state fermented products of *G. lucidum* GANO99 (5x), respectively (Figure 6(a)). These observations affected the Firmicutes/Bacteroidetes ratio (Figure 6(b)). The alpha-diversity index of the gut microbiota was assayed via Chao1, ACE, Shannon, and Simpson analyses (Figure 6(c)), with no difference observed in alpha diversity between the groups. Moreover, beta-diversity indices were evaluated using partial least squares-discriminant analysis plots and principal component analyses. The results indicated distinct wide distributions (sample dots of AD and 1x dose liquid-state fermented products of *G. lucidum* GANO99 group were located on the left, whereas those of the 5x dose liquid-state fermented products of *G. lucidum* GANO99 group were located on the right, and those of the WT group were located in the middle and lower region) in WT and AD mice with or without liquid-state fermented products of *G. lucidum* GANO99 treatment (Figure 6(d)). Overall, the administration of liquid-state fermented products of *G. lucidum* GANO99 affected the microbial community in AD mice.

Taxa analysis of the gut microbiota composition in mice using a heatmap (Figure 7(a)) showed reductions in the abundance of *Alistipes*, *Lachnospiraceae_UCG_006*, *Odoribacter*, *Marvinbryantia*, *Chlamydia*, *Candidatus saccharimonas*, *Bifidobacterium*, *Faecalibaculum*, *Erysipelatoclostridium*, *Parasutterella*, and *Butyricicoccus* and elevations in the abundance of *Bacteroides*, *Lachnoclostridium*, and *Lactobacillus* in AD mice compared with those in the WT mice. Moreover, AD mice administered liquid-state fermented products of *G. lucidum* GANO99 had distinct gut microbiome profiles compared with those of WT and AD mice. The probiotic *Bifidobacterium* was recovered in the liquid-state fermented products of the *G. lucidum* GANO99 (5x) treatment group.

Figure 7(b) displays the relative abundance histogram of the top 10 species in the genus level. The AD group (37.08%) had a higher relative abundance of *Lactobacillus* than did the WT group (18.16%). Conversely, the AD group had a lower relative abundance of *Lachnospiraceae_NK4A136_ group* and *Ruminococcaceae_UUG_014* (20.86% and 0.61%, respectively) than did the WT group (26.3% and 2.61%, respectively). In addition, the relative abundances of *Lachnospiraceae_NK4A136_ group* and *Ruminococcaceae_UUG_014* in the group treated with 5x (26.26% and 4.31%, respectively) and 1x liquid-state fermented products

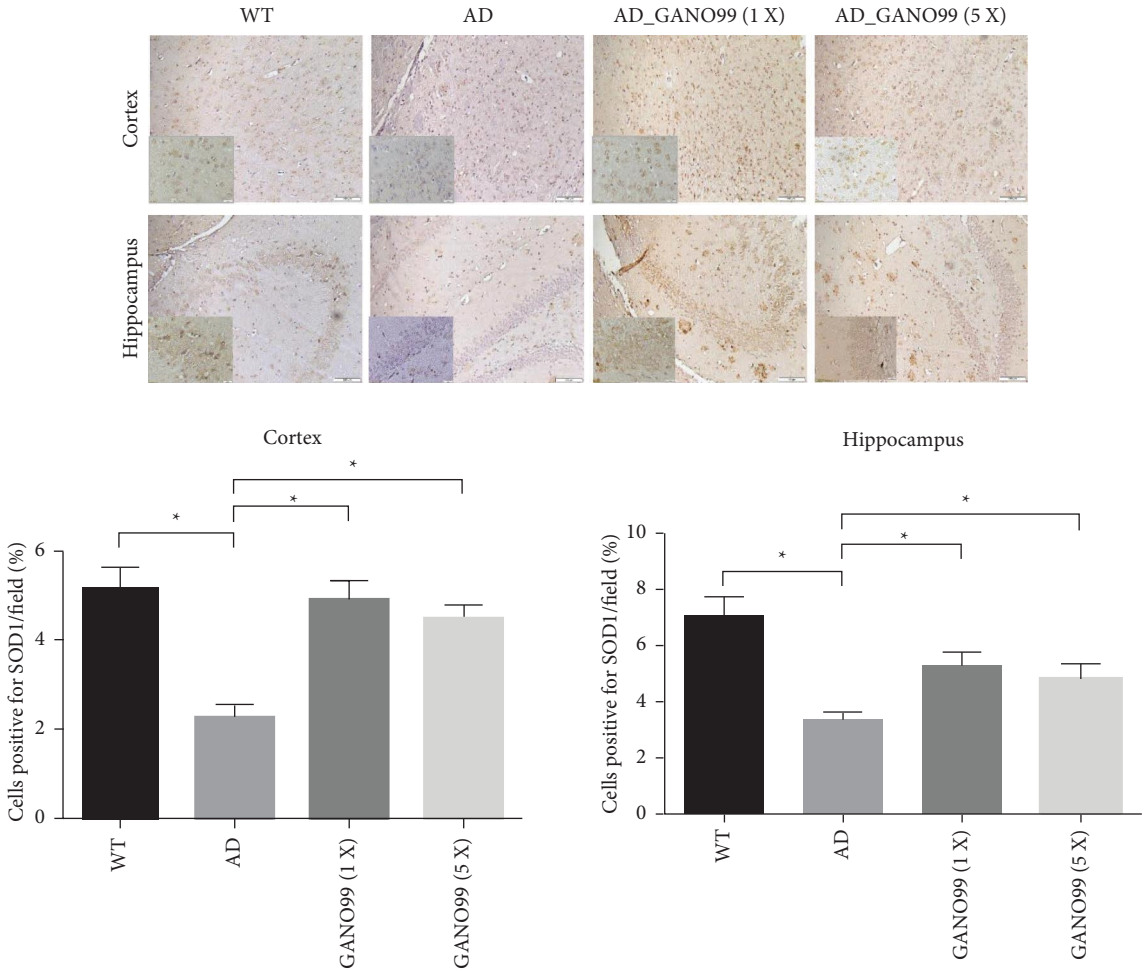


Figure 3: Effect of liquid-state fermented products of *G. lucidum* GANO99 on SOD expression in brains of C57BL/6 (WT) and $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. The positive cells were counted and values were averaged from 3 sections per brain. Eight animals per group were examined and the data are presented as mean ± SEM ($n = 6$). *Significant difference was determined by one-way ANOVA ($p < 0.05$).

of *G. lucidum* GANO99 (32.58% and 1.58%, respectively) were higher than those in the AD group.

An UpSet plot is an intersection analysis graph containing multiple data points. The upper bar graph represents the intersection size, indicating the number of OTUs in each mutually exclusive intersection. The lower left bar graph shows the set size, indicating the number of OTUs in each group (Figure 8(a)). Discriminative taxa were identified using LEfSe analysis at multiple levels for biomarkers through differential microbial identification. The results with the most difference (linear discriminant analysis score >3.0) were further investigated to understand the alterations in the microbiota structure. The AD group did not exhibit family enrichment. In contrast, the WT group exhibited enrichment of the order Bifidobacteriales, its affiliated family *Bifidobacteriaceae*, and the genera *Bifidobacterium*, *Faecalibaculum*, and *Desulfovibrio*. The liquid-state fermented products of *G. lucidum* GANO99 (1x) group exhibited an enrichment of the family_XIII_AD3011_group, whereas the liquid-state fermented products of *G. lucidum* GANO99 (5x) group exhibited enrichment of the genera *Ruminococcaceae_UUG_014*, *GCA_900066575,* and *Intestinimonas* (Figures 8(b) and 8(c)).

Figure 9 displays the microbial phenotype prediction of the microbiota analysis (box plots of the relative abundance of the species). The population of aerobic bacteria was elevated in AD mice administered the liquid-state fermented products of *G. lucidum* GANO99. The intestinal environment in AD mice administered the liquid-state fermented products of *G. lucidum* GANO99 was predicted. We discovered that intestinal microbes participated in fermentation and attenuation of the gut function in AD mice. However, the liquid-state fermented products of *G. lucidum* GANO99 acted as symbionts by reducing fermentation and chemo-heterotrophy in the gut.

4. Discussion

The newly introduced AD mice, $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, contain a humanized Aβ region with Swedish, Iberian, and Arctic mutations, which promote Aβ accumulation, a higher Aβ42/Aβ40 ratio, increased oligomerization, and decreased proteolysis triggering Aβ accumulation. Memory impairment in $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice occurs at 6 months of age [4]. In our study, memory protection and microbiota regulation

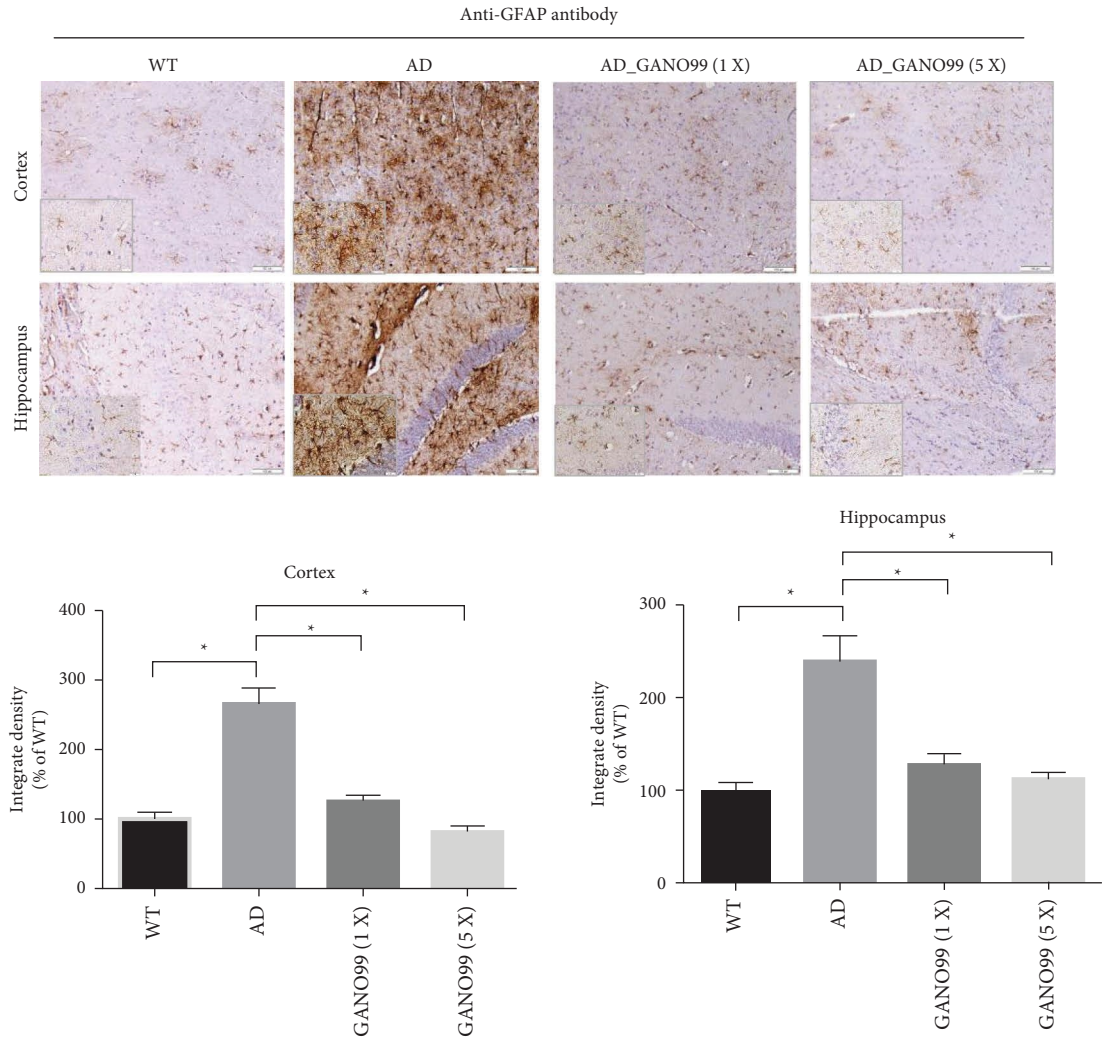


Figure 4: Effect of liquid-state fermented products of *G. lucidum* GANO99 on GFAP expression in brains of C57BL/6 (WT) and $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. The positive cells were counted and values were averaged from 3 sections per brain. Eight animals per group were examined and the data are presented as mean ± SEM ($n = 6$). *Significant difference was determined by one-way ANOVA ($p < 0.05$).

were evaluated in AD mice administered liquid-state fermented products of *G. lucidum* GANO99.

Behavioral experiments to evaluate memory deficits revealed that $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice exhibited memory impairments in the MWM and Y-maze tests. Memory function deficits may not be attributed to deficits in locomotor activity in the OFT. No significant difference was observed in motor capability between the WT and $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. In the MWM and Y-maze tests, $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice exhibited spatial memory deficits. Recent studies have reported that $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice exhibit definitive deficits in spatial learning and memory [20–22]. Our study discovered that liquid-state fermented products of *G. lucidum* GANO99 alleviated the impaired acquisition of spatial learning and memory. *G. lucidum* extract can increase spatial learning acquisition in the MWM test [23, 24].

The EPMT was used to evaluate the anxiety state of AD mice. Mice are curious and generally explore darker areas while avoiding light. This situation creates anxiety in the conflicting behaviors of asking and avoiding questions. Our study revealed that $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice spending more time and covering a greater distance in the open arm were indicative of lower anxiety levels, indicating that $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice exhibited robust anxiolytic-like behaviors [20, 25]. However, the anxiety state of $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice supplemented with high-dose *G. lucidum* GANO99 tended to be similar to that of WT mice but not to $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice supplemented with low-dose *G. lucidum* GANO99.

The pathological features of AD include neurofibrillary tangles, extracellular Aβ deposits, and neuroapoptosis [26, 27]. Astrocyte reactivity is an early feature of AD, and reactive astrocytes frequently surround neuritic plaques [4, 28, 29]. An increase in Aβ accumulation and GFAP activation in the brain have been reported in the AD mouse model and patients with AD [21]. In addition, we discovered that Aβ deposition in AD mice decreased significantly after treatment with liquid-state fermented products of *G. lucidum* GANO99. Moreover, elevated SOD levels may decrease Aβ accumulation and GFAP activation mediated by the liquid-state fermented products of *G. lucidum* GANO99.

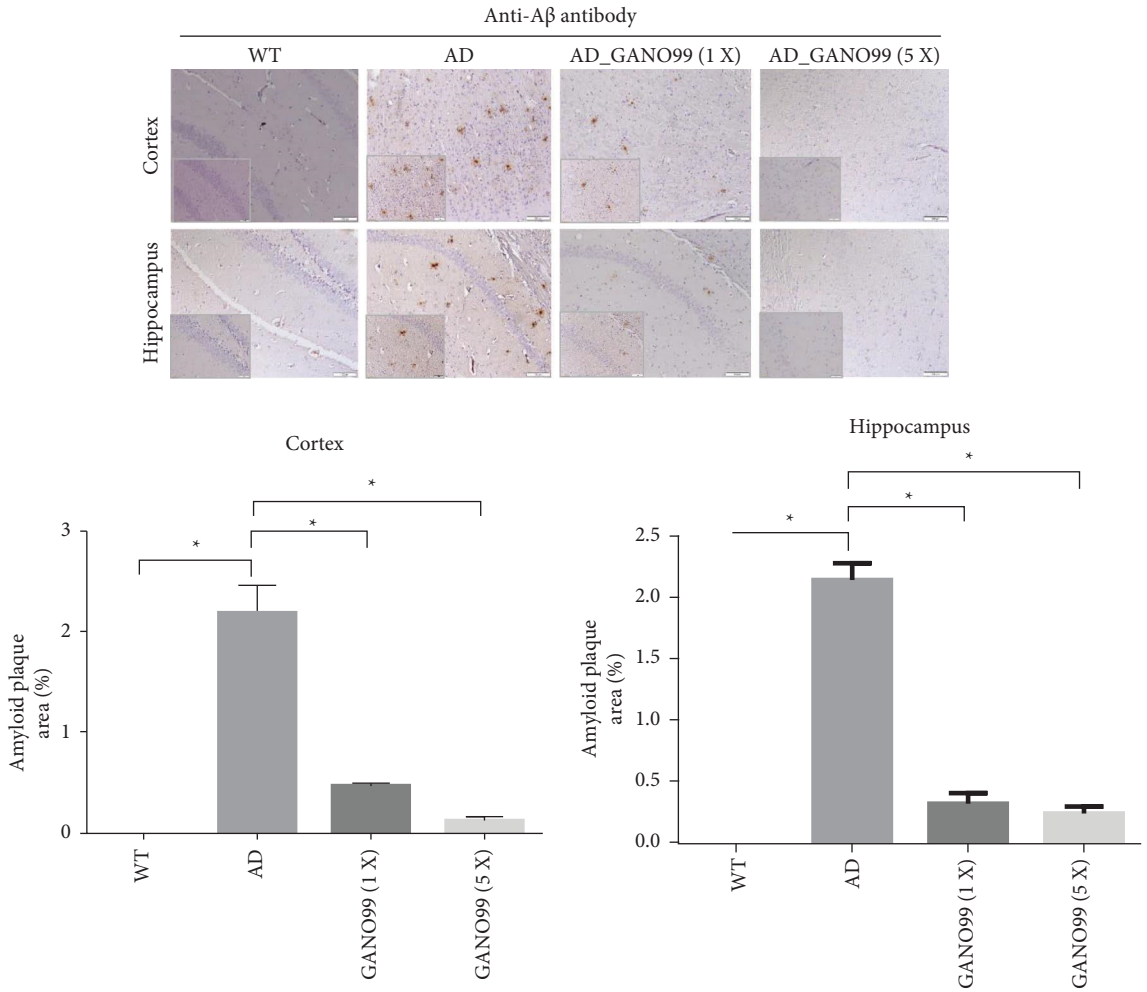
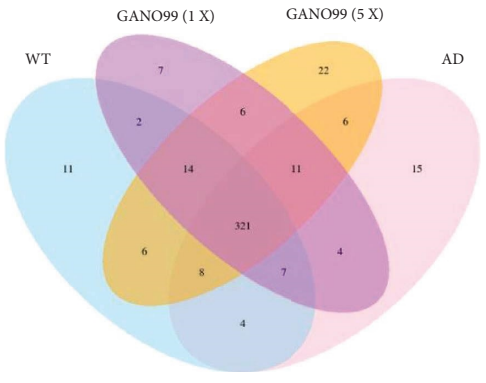


Figure 5: Effect of liquid-state fermented products of *G. lucidum* GANO99 on Aβ accumulation in brains of C57BL/6 (WT) and $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. The positive cells were counted and values were averaged from 3 sections per brain. Eight animals per group were examined and the data are presented as mean ± SEM ($n = 6$). *Significant difference was determined by one-way ANOVA ($p < 0.05$).



(a)

Figure 6: Continued.

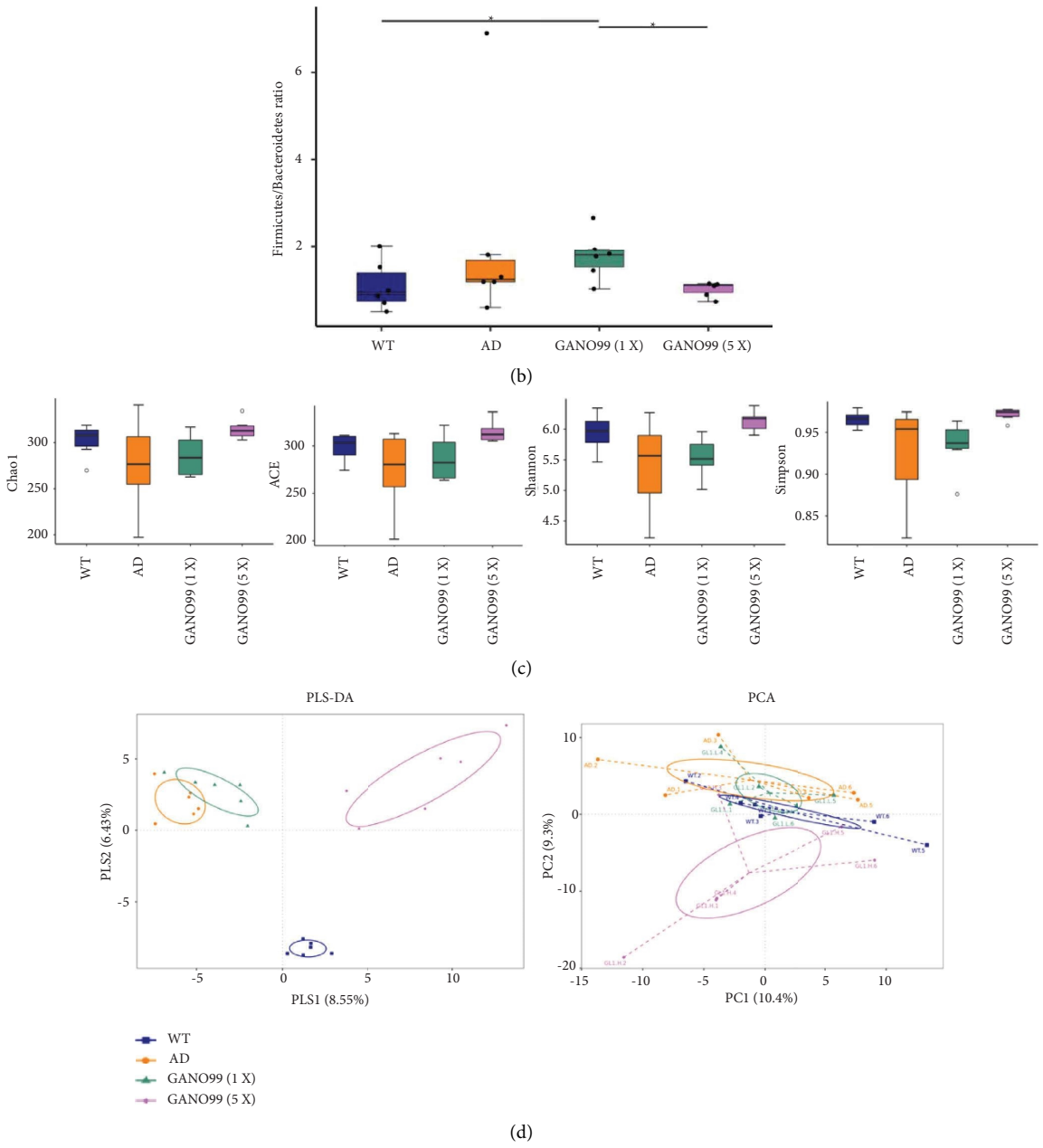
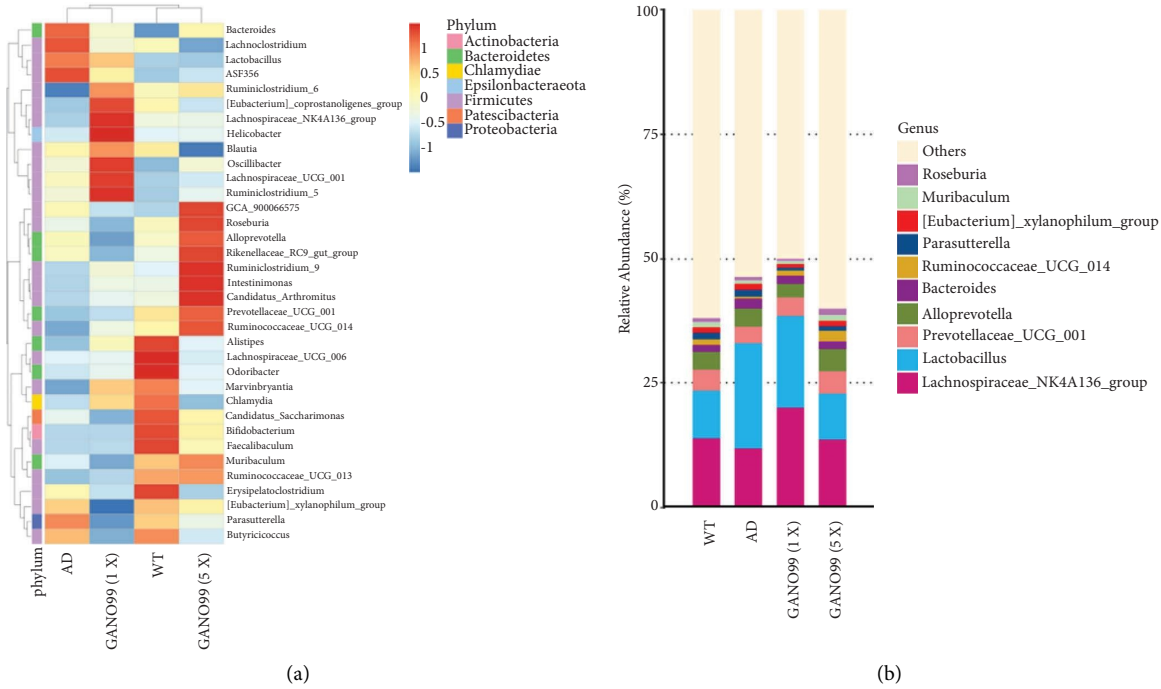


Figure 6: The microbial diversity of AD mice administered with the liquid-state fermented products of *G. lucidum* GANO99. (a) Venn diagram. The overlapping part indicates the number of OTUs. (b) The ratio of Firmicutes/Bacteroidetes of fecal microbes. (c) Alpha-diversity of gut microbiota by Chao1, ACE, Shannon, and Simpson statistical analysis. (d) Beta-diversity of gut microbiota by PLS-DA and PCA statistical analysis.

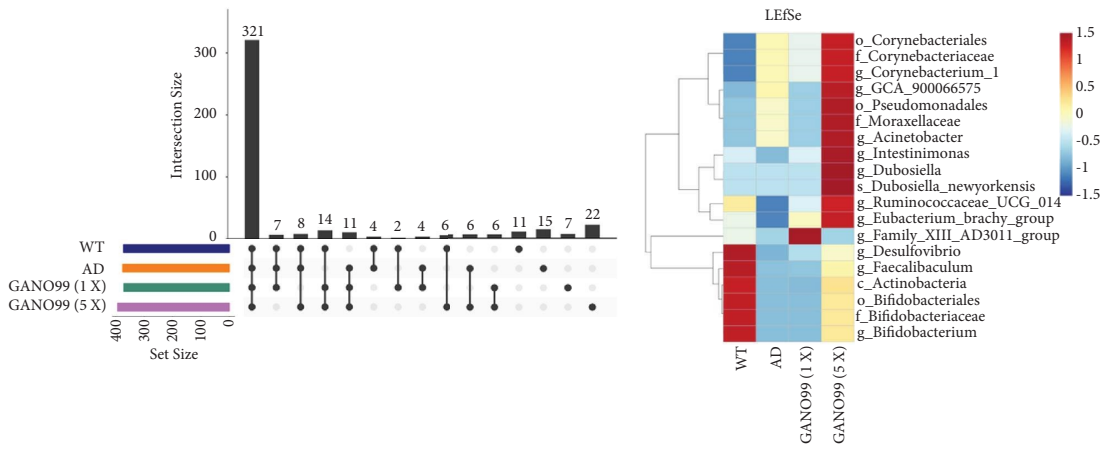
G. lucidum-related products can alleviate the pathological features of AD. A water extract of *G. lucidum* reduces Aβ accumulation and promotes neuronal proliferation in AD mouse brain [30]. *G. lucidum* alcohol extracts improve learning and memory function, ameliorate neuronal apoptosis and brain atrophy, and downregulate the expression of the AD intracellular marker $A\beta_{1-42}$ [31]. In addition, *G. lucidum* fruiting bodies ameliorate cognitive impairment, alleviate neuronal damage, and inhibit apoptosis in hippocampus tissues [32].

AD pathology may directly affect brain function and exacerbate cognitive deficits through alterations in the gut microbiota composition. AD pathology may be influenced by the gut microbiota, which plays a prominent role in the gut-brain axis [13]. The alteration in the gut microbiota of the mice in this study indicated that the gut microbiota composition tended to be similar between WT mice and the liquid-state fermented products of *G. lucidum* GANO99-treated AD mice. A gut-brain self-protective effect has also been reported in AD mice [13]. Recently,



(a) (b)

Figure 7: The microbiota changes of AD mice administered with the liquid-state fermented products of *G. lucidum* GANO99. (a) Heatmap of intestinal microbiota demonstrated the top 35 genera according to the abundance among all experimental groups. (b) Taxa analysis of gut microbiota composition in mice by relative abundance bar chart (top 10 classifications for genus level).



(a) (b)

Figure 8: Continued.

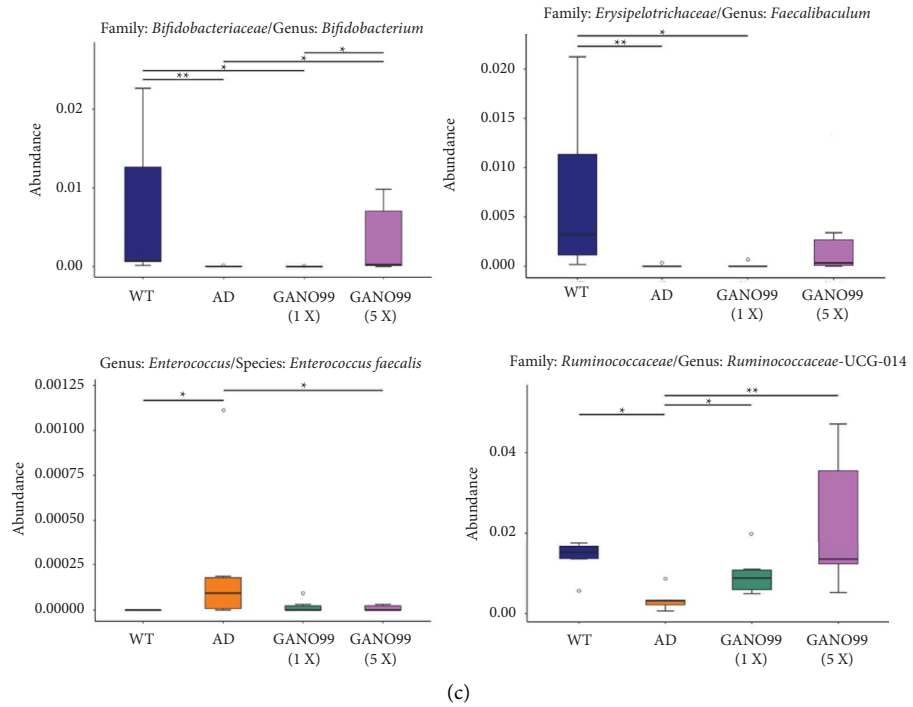


Figure 8: Differential microbial species in AD mice administered with the liquid-state fermented products of *G. lucidum* GANO99. (a) UpSet plot assay. (b) Biomarker compares abundance in different experimental groups by LEfSe assay. (c) Relative abundance (significant difference) of the gut microbiota in AD mice.

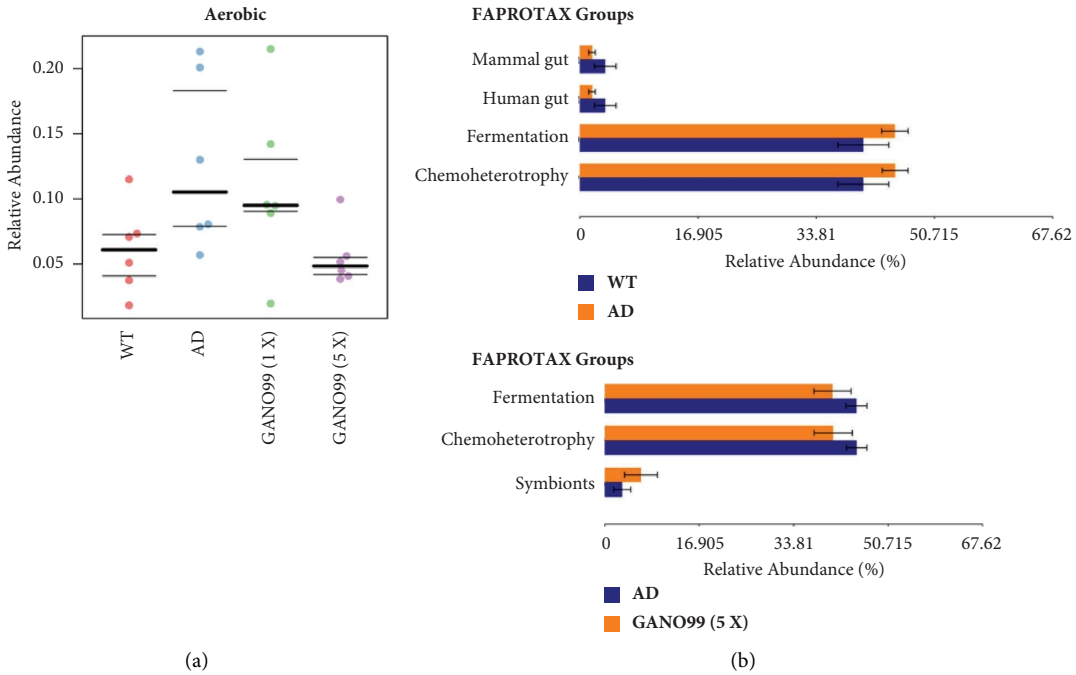


Figure 9: Microbial phenotype prediction of microbiota analysis. (a) The population of aerobic bacteria in AD mice administered with the liquid-state fermented products of *G. lucidum* GANO99. Box plots of relative abundance distribution of species. Three horizontal lines were quartiles, and each point represented one sample. (b) The prediction of intestinal environment in AD mice administered with the liquid-state fermented products of *G. lucidum* GANO99.

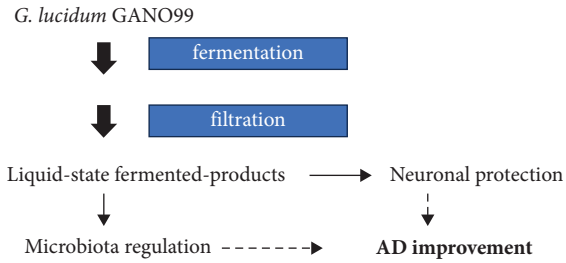


Figure 10: The potential mechanism of liquid-state fermented products of *G. lucidum* GANO99 against memory impairment of AD mice mediated by regulating gut microbiota.

the gut microbiota *Bifidobacterium* was shown to affect cognitive function through the main pathway involved in the gut-brain axis [33]. The liquid-state fermented products of *G. lucidum* GANO99 increased the abundance of *Bifidobacterium* species and improved cognitive function in both AD-like mouse models in this study. Therefore, *G. lucidum* GANO99 may be a promising therapeutic agent for regulating the gut commensal flora to alleviate AD.

5. Conclusions

For the first time, this study discloses the potential of liquid-state fermentation broth of *G. lucidum* GANO99 without mycelia in treating AD. These liquid-state fermented products proved effective in ameliorating memory impairments, inhibiting GFAP activation, reducing Aβ accumulation in the brain, decreasing oxidative damage in brain tissues, and ameliorating AD (Figure 10). Therefore, *G. lucidum* GANO99 may be a promising therapeutic agent for regulating the gut commensal flora to alleviate AD.

Data Availability

The *Ganoderma lucidum* GANO99 strains used to support the findings of this study were supplied by Syngen Biotech Co. Ltd. under license and so cannot be made freely available. Requests for access to the material should be made to Wei-Jen Chen via e-mail chen.william@standard.com.tw. In addition, the microbiota data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

This study was funded by Syngen Biotech Co. Ltd. to T.-L. Shen. Nervertheless, Bao-Hong Lee and Tang-Long Shen declare no conflicts of interest. The remaining authors, Wei-Jen Chen, Yu-Shan Wei, Hsin-I Lo, Hui-Fang Chu, and Ming-Chung Tseng, are currently employees in Syngen Biotech Co. Ltd., and Wei-Jen Chen possesses the stock of the company. In fact, the intellectual property derived from the current study has not been claimed.

Acknowledgments

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