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Cordyceps cicadae NTTU 868 Mycelium with The Addition of Bioavailable Forms of Magnesium from Deep Ocean Water Prevents the Aβ40 and Streptozotocin-Induced Memory Deficit via Suppressing Alzheimer's Disease Risk Factors and Increasing Magnesium Uptake of Brain

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Abstract: Alzheimer's disease (AD) is a common neurodegenerative disease characterized by continuous accumulation of β-amyloid (Aβ) in the brain. Deep ocean water (DOW) with rich inorganic salts and minerals was proven to promote fungi growth and metabolism. *Cordyceps cicada*, a functional food fungus, can produce higher anti-oxidant and anti-inflammatory compounds including adenosine, polysaccharide, and N(6)-(2-Hydroxyethyl) adenosine (HEA). This study used DOW as the culture water of *C. cicadae* NTTU 868 for producing DOW-cultured *C. cicadae* (DCC), and further investigated the effects and mechanisms on improving the memory deficit and repressing risk factors expressions in Aβ40 and streptozotocin (STZ)-induced Alzheimer's disease rats model. In the results, DCC including mycelium and filtrate had adenosine, HEA, polysaccharide, and intracellular Mg^{2+} after fermentation with DOW. DCC had more effect on the improvement of memory deficit because it suppressed Aβ40 and streptozotocin (STZ) infusion caused BACE, pro-inflammatory factors expressions, and Aβ40 accumulation by increasing sRAGE expression in the brain. Furthermore, DCC enhanced the MAGT1 expression due to high organic magnesium, which can reverse Aβ40-induced cortex magnesium deficiency and further repress Aβ40 accumulation.

Keywords: deep ocean water; *Cordyceps cicadae*; Alzheimer's disease; β-amyloid; polysaccharide; N(6)-(2-Hydroxyethyl) adenosine)

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1. Introduction

Cordyceps cicadae, a Chinese traditional food fungus, has antioxidation, anti-inflammation [1], blood sugar regulation (Li et al., 2018), renal function improvement [2,3], liver protection [4], and neuroprotection [5] properties. The functional components of *C. cicadae* are adenosine, N(6)-(2-Hydroxyethyl) adenosine (HEA), and polysaccharides [6,7], which are proven to have antioxidation, anti-inflammatory, and anti-aging properties [8].

Researchers have verified that deep ocean water (DOW) can treat obesity [9] and cardiovascular diseases [10] and can fight atherosclerosis [11]. Furthermore, when DOW is applied to microbial fermentation, the nutrient content can promote the production of *Antrodia camphorata* mycelium and increase the quantity and anti-oxidation effects of its ingredients (triterpenoids, polysaccharides, and total flavonoids) [12], drive production of the functional components in *Monascus* (monascin and ankaflavin), lower the content of the toxin citrinin in kidneys and the liver [13], and increase the content of adenosine and cordycepin in *Cordyceps* fermentation products [14]. It can also increase the therapeutic effects of microbial fermentation products on diseases. Furthermore, the main ions in DOW are absorbed by microorganisms during the microbial fermentation process and are then converted to be better absorbed by the human body [15].

C. cicadae is known to have antioxidative, anti-inflammatory, and neuroprotective effects, and DOW nutrients not only treat diseases but also effectively increase the content of the functional ingredients in microbial fermentation products and thereby improve disease treatment effectiveness. During the microbial fermentation process, DOW nutrients are absorbed and utilized effectively by microorganisms; this effect allows DOW nutrients to have higher bioavailability, which endows microbial fermentation products with greater synergistic health benefits. This study is an investigation of DOW-cultivated *C. cicadae* products and their therapeutic effects on Alzheimer's disease (AD) as well as an investigation into whether $MgCl_2$ in deep ocean water is the main functional mineral increasing DOW fermentation of *C. cicadae* products. The study simultaneously explored the main mechanism through which DOW, HEA, and polysaccharides have effects on overall AD improvements.

2. Materials and Methods

2.1. Chemicals

N(6)-(2-Hydroxyethyl) adenosine (HEA) (>95% purity) and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Difco Co. (Detroit, MI, USA). Ethanol (95%) was purchased from Taiwan Tobacco and Liquor Co. (Taipei, Taiwan). Aβ40 was purchased from Tocris Bioscience Co. (Ellisville, MO, USA). Mouse TNF-α protein (50349-MNAE), mouse IL-6 protein (50349-MNAE), mouse IL-1β protein (50349-MNAE), and rat iNOS protein (Q06518) were purchased from SinoBiological Inc. (North Wales, PA, USA). Rabbit iNOS antibody (FNab04325) was purchased from Cloud-Clone Corp. (Wuhan, China). Rabbit anti-mouse TNF-α polyclonal antibody (AB2148P) were purchased from EMD Millipore Corporation (Temecula, CA, USA). Mouse anti-rat IL-6 monoclonal antibody (sc-57315) and mouse anti-human IL-1β monoclonal antibody (sc-32294) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat anti-rabbit IgG, (H+L), peroxidase conjugated anti-body (31460) were purchased from Pierce Biotechnology (Rockford, IL, USA)

2.2. The Source of DOW

The concentrated DOW provided from the Eastern Taiwan Deep Sea Water Innovation and Research Center (Taitung, Taiwan) was pumped from a depth of 670 m in the Pacific Ocean near the Eastern Taiwan and processed though the electrodeionization and vacuum concentration. According to our previous study, DOW including 20.65 mg/L Mg^{2+} was defined as onefold DOW. In this study, 15-fold DOW (including 309.75 mg/L Mg^{2+}) was prepared by the dilution of concentrated DOW (including 82,640 mg/L Mg^{2+}) with UPW. The concentrations of the trace elements and minerals in 15-fold DOW included 309.75 mg/L Mg, 24.08 mg/L Na, 36.56 mg/L K, 104.95 mg/L Ca, 1.237 μg/L Zn, 2.436 μg/L Cu, 0.441 mg/L Sr, 0.375 μg/L Mo, 8.4 mg/L H_2SiO_3, 1.65 mg/L phosphate, and 0.949 g/L chloride.

2.3. Sample Preparation

C. cicadae NTTU 868 fermentation product was obtained from 1.5-L of submerged fermentation. *C. cicadae* NTTU 868 was cultured with potato dextrose broth at 23 °C for 3 days for the seed culture, and then inoculated (10%) into the fermentation broth (containing 2.4% potato dextrose broth powder and 0.2% yeast extract in 1.5 L ultra-pure water, DOW, or $MgCl_2$, pH 6.8) and cultured at 23 °C for 10 days. Whole submerged fermentation product including mycelium and filtrate were homogenized for the preparation of animal test sample.

The mycelium was separated from whole submerged fermentation product through centrifugation. Next, the mycelium was dried by using the freeze dryer. The dried *C. cicadae* NTTU 868 mycelium was extracted in ultra-pure water solution at 95 °C for an hour and then centrifuged for 10 min at 8000× *g* to get the supernate. The filtrate was collected for

the analysis of extracellular polysaccharides. The supernate and filtrate were treated with 3 times the volume of 95% ethanol and extracted at 60 °C for 60 min in order to deposit intracellular and extracellular polysaccharides. The After centrifugation (8000× *g*, 10 min), the polysaccharides depositions were collected for the use of animal test sample.

2.4. Extract and Quantitative Analysis of Adenosine, HEA, and Polysaccharides

Dried *C. cicadae* NTTU 868 mycelium were extracted using 10 volumes of 20% methanol at 60 °C for 30 min and vortexed at every 10 min intervals. The supernatant was collected by centrifugation at 8000× *g* for 10 min. The extract was stored at −20 °C overnight and centrifuged at 8000× *g* for 10 min to remove polysaccharides [16]. Adenosine, HEA were determined by high performance liquid chromatography (HPLC) with a reverse-phase column (Mightysil RP-18 GP 5 µm C18, 250 × 4.6 mm, Kanto Chemical Co., Inc., Tokyo, Japan) and diode array detector (DAD, L-2000 series, Hitachi, Japan). The mobile phase (A solvent: methanol; B solvent: water) was eluted with 0.8 mL/min of flow rate and gradient condition (A solvent: methanol; B solvent: water; 0–3 min, 10% A; 4–8 min, 0% A; 8–18 min, 0% A to 100% A; 18–25 min, 10% A, 28 to 30 min). A 20 µL sample was injected each time. Absorption spectra of eluted compounds were recorded at 262 nm [1]. The polysaccharide was resolved to a suitable concentration by distilled water. The polysaccharide concentration of filtrate was analyzed according to the previous study [17]. The magnesium ion of mycelium extract was determined by ICP-OES (Perkin Elmer Optima 2100 DV, Waltham, MA, USA)

2.5. Animals Grouping and Experiment Schedule

Male Sprague Dawley (SD) rats at 6-8 weeks of age were purchased from the BioLasco Co. (Taipei, Taiwan). The animals were housed individually and allowed free access to a standard laboratory chow (Ralston Purina, St Louis, MO, USA) and water. They were kept in a temperature controlled room (23 °C) under a 12L:12D cycle (light on at 6:00) and were given free access to food and water. In the experiment, the 48 rats were randomly divided to 8 groups and fed standard chow (control group, NOR; 4.5% fat, 3.34 kcal/g).

Rats were infused with vehicle solution (Vh group) or Aβ40 and streptozotocin (STZ) solution (Aβ group) by intracerebroventricular (i.c.v.) injection without administration of test materials. Other rats with i.c.v Aβ40 and STZ infusion were administered DOW (0.0976 mL/kg/day, DOW group), Ultra pure water-cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, HEA group), or polysaccharides (1.5 mg/kg/day, PS group). The recommendation dosage of homogenate of *C. cicadae* NTTU 868 fermented product is suggested as 250 g/day for 60 kg adult. Wet UCC, DCC, and MgCC homogenate (250 g) were freeze died, and obtained 1.93 g, 2.12 g, and 1.31 g dry products. The doses of the test substances used in this study were calculated in accordance with Boyd's Formula of Body Surface Area as recommended by the FDA (Food and Drug Administration) [18,19]. Therefore, UCC of 200 mg/kg B.W. /day, DCC of 220 mg/kg B.W. /day, MgCC of 137 mg/kg B.W./day for animal dosages were corresponding to the human dosage that a 60 kg adult daily intake 250 g homogenate of *C. cicadae* NTTU 868 fermented product. The contents of DOW, HEA, and polysaccharide of DOW group, HEA group, and PS group were equal to that in DCC group, respectively.

The experiment schedule of AD animal model was shown as follow. Aβ40 infusion on 0th day was continued for 28 days and the test substance in suspension was orally administrated to the rat from the 1st day to the 28th day. The behavioral test was started on the 19th day. The passive avoidance task was carried out from 19th day to the 21st day. Subsequent reference memory task, probe test, and working memory task were started on the 22nd day, the 24th day, and the 25th day. On the 28th day, rats were sacrificed, and the brain tissues were collected for the measurement of TNFα, IL-6, IL1β, sRAGE, *p*-tau, BACE, sAPPα, Aβ40, and MAGT1 protein expressions.

2.6. Surgery for i.c.v. Aβ40 Infusion

Rats were anesthetized with sodium pentobarbital (50 mg/kg BW i.p.). The left skull was exposed and drilled (relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) according to the atlas of Paxinos and Watson [20] using a stereotaxic frame (Narishige, Tokyo, Japan). Aβ40 was prepared in the vehicle solvent of 35% (*v*/*v*) acetonitrile plus 0.1% (*v*/*v*) trifluoroacetic acid (pH 2.0). The osmotic mini-pump (2004, Durect Co., Cupertino, CA, USA) used to result in an animal model of AD with impaired memory was filled with AD solution (24.299 μg Aβ40 and 0.9 mg STZ in 180 μL) or the vehicle solution. The outlet of infusion cannula was inserted 4.0 mm into the left ventricle and attached to the skull with dental cement, and then the mini-pump was quickly implanted into the backs of the rats. AD solution of 180 μL contained in the osmotic pump was continuously infused into left ventricle by 0.28 μL/h for 28 days [21].

2.7. Apparatus of Water Maze

The Morris water maze task was used to evaluate the memory and learning ability from the 22nd day to the 27th day [21]. A black circular tank (diameter: 140 cm, height: 45 cm) was used as the apparatus of water maze in which a movable escape platform (diameter: 10 cm, height: 25 cm) was located inside the tank. The tank was filled to a height of 27.5 cm with water of temperature approximately 23 °C; thus, the surface of the platform was 2.5 cm below the surface of the water. The circular tank was divided into four quadrants (I, II, III, and IV), and a position with equal distance from center and edge in the middle of each quadrant was marked for the location of platform. The water tank was located in a test room with many cues external to maze. The room had adjustable indirect light, and camera was set at ceiling above the center of water tank. The position of the cues remained unchanged throughout the water-maze task.

2.8. Morris Water Maze Task

According to the procedure of our previous study [21], reference memory test was carried out from the 22nd day to the 24th day and included continuous 4 trials per day. Probe test was immediately carried out after the 12th training trial of reference memory task on the 24th day. Working memory test was performed from the 25th day to the 27th day and consisted of five trails per day.

2.9. Preparation of Brains

After completing the behavioral studies, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.), and the blood was collected; the cerebral cortex and hippocampus were separated from the whole brain on ice, blotted gently with filter paper to remove blood and extraneous tissue fragments, then flash-frozen with liquid nitrogen and stored at −80 °C until use. Hippocampus and cortex tissues (100 mg) were crushed with an amalgam mixer (UT-1600, Sharp, Osaka, Japan) and suspended in 1.0 mL of ice-cold Tris saline (50 mM Tris-HCl, pH, 7.6, 0.15 M NaCl) buffer containing 1% (*v*/*v*) Triton X-100 and protease inhibitor cocktail, and then sonicated for 30 sec. The homogenate was centrifuged at 100,000× *g* for 30 min and the supernatant was used for magnesium analysis using the commercial kit (Fortress Diagnostics Ltd., Antrim, UK). Regarding the protein extraction for immunoblotting, the tissue (100 mg) was homogenated in 1.0 mL of lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 0.2% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, and 1 mM Na_3VO_4) and brief sonication (10 s). The homogenate was centrifuged at 100,000× *g* for 30 min and the supernatant was used for immunoblotting assay.

2.10. Enzyme-Linked Immunosorbent Assay

TNF-α, IL-1β, and IL-6 were determined by self-coating ELISA kit (DY008, R&D Systems, Inc., Minneapolis, MN, USA). TNF-α, IL-1β, and IL-6 recombinant proteins were used as the standards. The standards or tissue homogenates were added to a 96-

well plate at 37 °C for 90 min (using a microplate spectrophotometer) (Thermo Fisher Scientific Inc., Waltham, MA, USA). After removing the standard or liver homogenates, primary antibodies were added and incubated at 37 °C for 60 min and then aspirated and washed 3 times by filling each well with 350 µL of fresh wash buffer. The secondary antibodies were added and incubated at 37 °C for 30 min. After washing 6 times, color subtract (30% H_2O_2 and TMB reagent 1:1) was added in each well and incubated at 37 °C for 15 min. Subsequently, 2N H_2SO_4 as stop solution was added and the plate read at 450 nm immediately.

The other proteins including Aβ40 (ER0754, BACE (ER0756), sRAGE (MBS9338787), were determined by commercial ELISA kits (Fine Biotech Ltd., Wuhan, China). sAPPα (27419) were determined by commercial ELISA kits (Immuno Biological Laboratories Inc, Minneapolis, MN, USA). MAGT1 (MBS9338787) were determined by commercial ELISA kits (Mybiosource Inc., San Diego, CA, USA).

2.11. Statistical Analysis

Data are expressed as means ± standard deviation. Analysis of variance by Duncan's test and Pearson's product-moment correlation coefficient test were determined using SPSS version 10.0 software (SPSS Institute, Inc., Chicago, IL, USA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of DOW and $MgCl_2$ on C. cicadae and the Production of Functional Components

DOW is abundant in nutrients and minerals. Studies have verified that cultivating *Monascus*, *Cordyceps militaris*, and *Antrodia cinnamomea* with DOW can effectively increase their mycelium growth and functional component content [13–15,22]. Table 1 compares the effects of UPW, DOW, and $MgCl_2$ solutions on *C. cicadae* and the production of their functional components on a 1.5 L cultivation scale. Compared with the other two groups, UPW-cultivated *C. cicadae* has lower HEA but exhibits no significant differences in total polysaccharides. However, DOW can significantly increase *C. cicadae* mycelium content and HEA product even though the highest $MgCl_2$ solutions content lowered biomass and adenosine content while significantly increasing HEA content ($p < 0.05$). In addition, Intracellular Mg^{2+} concentration of mycelium cultured by DOW or $MgCl_2$ was more than that by UPW, suggesting that the mycelium bio-absorbs the magnesium ions in DOW or $MgCl_2$ solution. Furthermore, Mg^{2+} may be converted into organic ions after being absorbed in cells.

Table 1. Effect of DOW and $MgCl_2$ solution on the production of dry mycelium and functional compounds of *C. cicadae* NTTU 868 (fermented capacity: 1.5 L).

Culture Water	Dry Mycelium (g/L)	Adenosine (mg/L)	HEA (mg/L)	Intracellular Polysaccharide (mg/L)	Extracellular Polysaccharide (mg/L)	Intracellular Magnesium (mg/L)
UPW	7.27 ± 0.47^{b}	7.15 ± 0.94^{b}	0.88 ± 0.08^{a}	99.3 ± 8.7^{b}	548 ± 104^{a}	10.1 ± 0.9^{a}
DOW	8.07 ± 0.73^{c}	6.67 ± 0.48^{b}	1.04 ± 0.15^{a}	88.0 ± 12.0^{ab}	518 ± 141^{a}	161.4 ± 12.0^{c}
$MgCl_2$	5.27 ± 0.59^{a}	1.58 ± 0.05^{a}	1.32 ± 0.15^{b}	74.7 ± 14.7^{a}	554 ± 131^{a}	132.0 ± 14.7^{b}

The data are presented as mean ± SD ($n = 3$). ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$). UPW: ultrapure water, DOW: deep ocean water, $MgCl_2$: magnesium chloride. HEA: N(6)-(2-Hydroxyethyl) adenosine.

3.2. Effects of DOW-Cultivated C. cicadae Fermentation Products on the Memory Tests and Spatial Learning of Rats with Brain Infusions of Aβ40 and STZ

This experiment involved infusions of Aβ40 and STZ to induce memory loss in rats and exploration of the effects of *C. cicadae* fermentation products cultivated with DOW on brains infused with Aβ40 and STZ, to treat AD. The main effects of DOW and

C. cicadae on AD improvements were explored through the comparison of water cultures and ingredients.

The reference memory tests were conducted by placing a hidden platform in the IV quadrant and recording the amount of time the rats spent searching the platform each time. This was one of the approaches to evaluating the learning and memory of the rats. The results are presented in Figure 1. On the first day, no significant differences were present in the amount of time spent searching the platform by each group of animals ($p > 0.05$), whereas the training results from the second and third day demonstrated that compared with the Vh group, which had normal memory, the Aβ group spent more time and exhibited significant differences ($p < 0.05$). Except the D group, which displayed a downward trend, the groups with other test substances exhibited reduced time spent on the reference memory test. The third day's results indicated that the DCC group exhibited the most satisfactory results, with significantly reduced time spent searching the platform ($p < 0.05$), and therefore demonstrated the most satisfactory improvement of long-term memory.

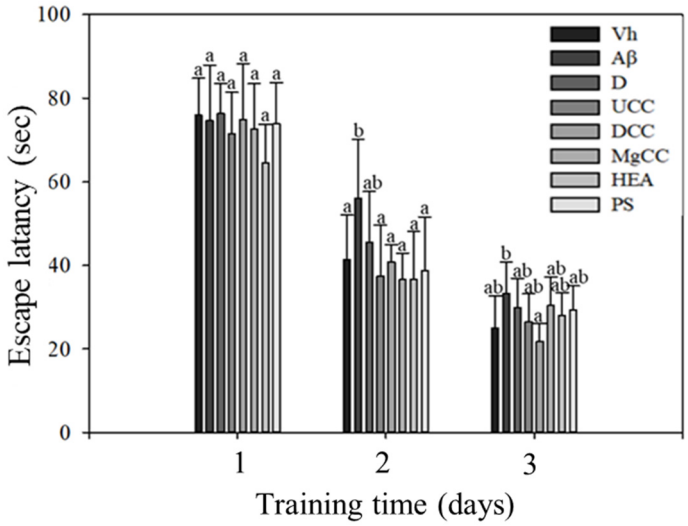

Figure 1. Effect of various *C. cicadae* NTTU 868 fermented products on the searching escape latency of rats with Alzheimer's disease (AD) induced by Aβ40 and streptozotocin (STZ) i.c.v infusion in a reference memory task. Rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

After the reference memory test was completed, the platform was removed to conduct the probe test. The swimming pathway is beneficial in identifying the authenticity of the learning and memory of lab animals during spatial exploration tests. After removing the hidden platform, the time and pathway distance of the animals roaming where the platform was originally placed and the average swimming speed of the rats within the space were viewed as the memory and learning indicators of the spatial exploration tests. The results are presented in Figure 2a. The Aβ group searched for the target quadrant without direction and without aim, and their swimming pathway covered the entire pool. Conversely, the Vh, D, UCC, DCC, MgCC, HEA, and PS groups swam directly to the target quadrant and spent considerable time moving back and forth in the target quadrant. Furthermore, as Figure 2b demonstrates, the Vh, DCC, MgCC, and PS groups spent more

time going back and forth in the target quadrant ($p < 0.05$) than did the Aβ group, by 64%, 54.7%, 29.9%, and 28.9%, respectively; the D, UCC, and HEA groups spent 8.17%, 20.85%, and 11.92% more time.

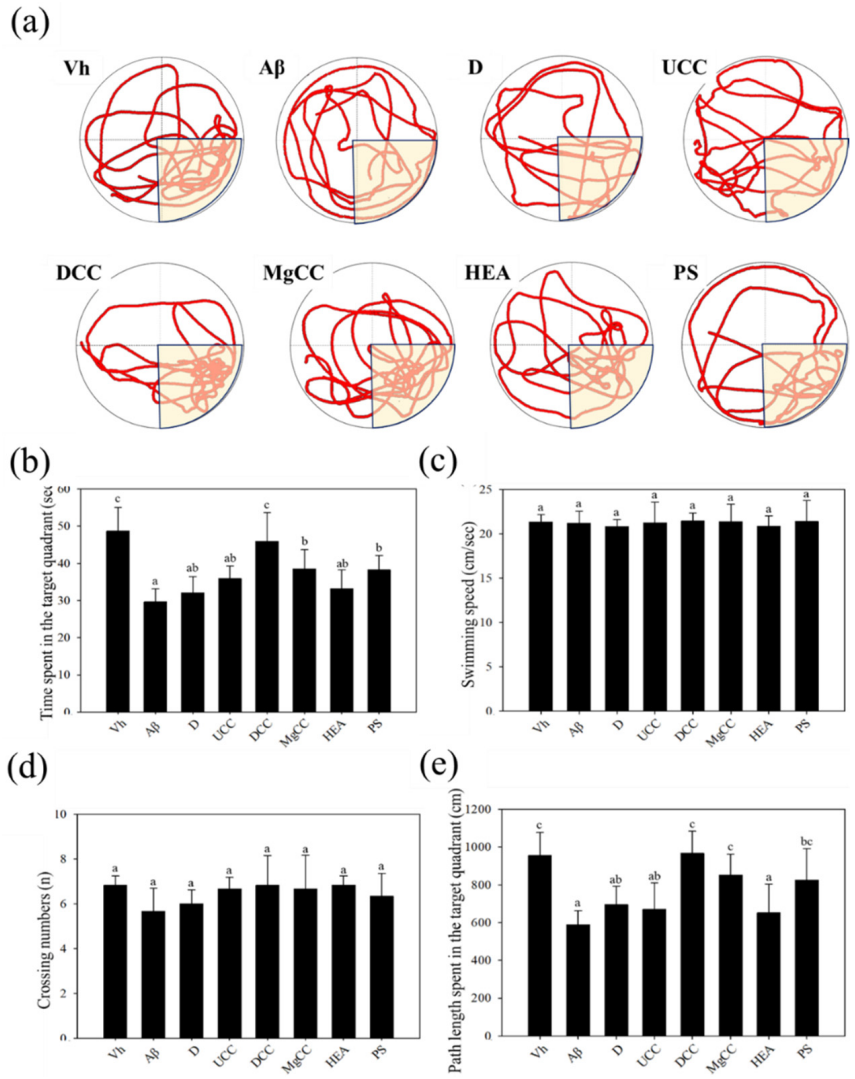

Figure 2. Effect of various *C. cicadae* NTTU 868 fermented products on the memory and learning of rats with AD induced by Aβ40 and STZ i.c.v infusion in spatial probe traces. (**a**) Swimming track (**b**) Time spent in the target quadrant (**c**) Swimming speed (**d**) Path length in the target quadrant (**e**) Crossing numbers. Groups of rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

In addition to swimming time, the speed, number of times entering the quadrant, and swimming distances were analyzed. As can be observed in Figure 2c, no significant differences were present in the swimming speed of the groups; accordingly, swimming speed did not affect the length of the pathways lingering in the target quadrant. Figure 2d presents the number of times the rats entered or passed through the target quadrant.

Although the differences between groups were nonsignificant ($p > 0.05$), the Aβ group was observed to have fewer entries into the target quadrant, whereas the DCC group exhibited more entries. Compared with the Aβ group, the D, UCC, DCC, MgCC, HEA, and PS groups had 20.5%, 5.88%, 17.6%, 20.5%, 17.6%, 20.5%, and 11.7% more entries.

The swimming pathways of the rats entering or passing through the target quadrant are presented in Figure 2e. The Vh, DCC, MgCC, and PS groups had longer pathways moving back and forth within the target quadrant, which were respectively 62.2%, 64.2%, 44.8%, and 40% longer than the Aβ group's pathway ($p < 0.05$); the D, UCC, and HEA groups had 17.9%, 13.9%, and 10.8% longer pathways. These results are similar to the trend in Figure 2b, with the Vh, DCC, MgCC, and PS groups exhibiting significantly higher results than the Aβ group ($p < 0.05$). Therefore, given the lack of significant differences in swimming speed ($p > 0.05$), the time spent going back and forth in the target quadrant and the length of the pathway were similar, verifying that being fed DCC, MgCC, and PS can effectively improve the spatial learning and memory of rats with AD.

3.3. C. cicadae Fermentation Products and the Expression of Aβ40 and BACE Proteins in the Hippocampus of Rats with Brain Infusions of Aβ40 and STZ

After being cleaved by BACE and γ-secretase, amyloid precursor protein (APP) transforms into Aβ [23]. Increased BACE expression or enzyme activity in the brain drives Aβ production in the brain and aggravates abnormal phosphorylation of tau proteins to form neural tangles [24]. Therefore, in this study, the expression Aβ40 and BACE proteins in the hippocampus of rats with AD were assessed.

Figure 3a,b present the amount of expressed AD factors Aβ40 and BACE. Brain infusions of Aβ40 and STZ significantly increased the expression of Aβ40 and BACE proteins ($p < 0.05$). Although DOW (D group) cannot reduce Aβ40 and BACE protein expressions in the hippocampus ($p > 0.05$), the DCC group had the best results with substantially suppressed Aβ40 and BACE content. This demonstrates that DOW-cultivated DCC has the ability to reduce the expression of AD risk factors and has better results than UCC, MgCC, HEA, or polysaccharides. This effect may derive from the additive effects formed by various ingredients.

After α-secretase cleaving, APP produces sAPPα and C83. Particularly, sAPPα elevates the formation of myelin, increasing the neurotransmission of the nervous system [25]. As Figure 3c demonstrates, infusions of Aβ40 and STZ slightly lower the expression of sAPPα; of all the test groups, only the DCC group exhibited a slight upward trend ($p < 0.05$).

The tau protein is a microtubule-associated protein of mature neurons. Excessive phosphorylation produces neural tangles, resulting in impaired transmission of neural signals and affecting learning and memory [26,27]. As Figure 3d indicates, *p*-tau expression was significantly increased as a result of Aβ40 and STZ infusions ($p < 0.05$), but the D, UCC, DCC, and HEA groups did not exhibit lower *p*-tau ($p < 0.05$). The MgCC group exhibited significantly reduced *p*-tau expression ($p < 0.05$).

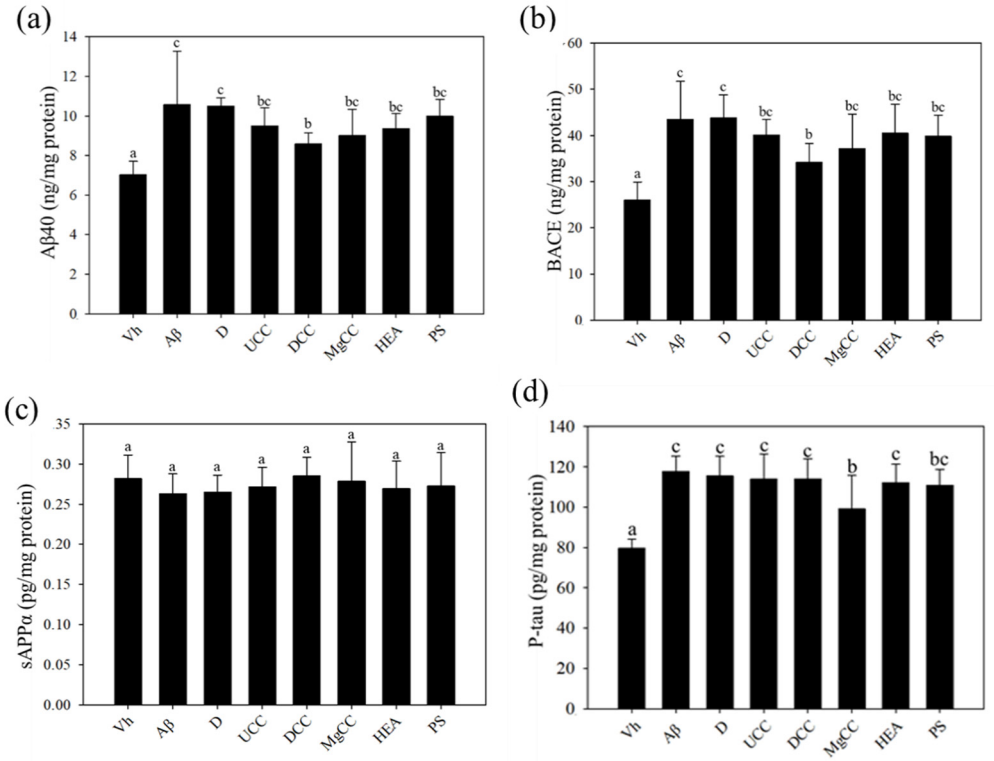

Figure 3. Effect of various *C. cicadae* NTTU 868 fermented products on Aβ40 (**a**), BACE (**b**), and sAPPα (**c**) and p-tau (**d**) protein expression of rats with AD induced by Aβ40 and STZ i.c.v infusion in the hippocampus. Groups of rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

3.4. Effects of C. cicadae Fermentation Products on the Expression of TNF-α, IL-6, and IL-1β in the Hippocampus of Rats with Brain Infusions of Aβ40 and STZ

Excessive accumulation of Aβ in the brain activates stellate cells and macrophages to produce large amounts of proinflammatory factors (TNF-α, IL-6, and IL-1β) and ROS. This reaction results in damage to and inflammation of neuronal cells and even the apoptosis of neurons [28,29]. Figure 4 presents the amount of TNF-α, IL-6, and IL-1β expressed in the cortex and hippocampus. Expressions of TNF-α, IL-6, and IL-1β in the cortex and hippocampus demonstrate similar trends—that Aβ and STZ can induce significantly higher expressions of TNF-α, IL-6, and IL-1β ($p < 0.05$). DCC performs more favorably than UCC and MgCC in reducing the expression of TNF-α, IL-6, and IL-1β. However, by itself, DOW did not result in significant improvements ($p < 0.05$). This demonstrates that by itself, DOW does not have a significant effect in resisting inflammation of brain tissue induced by Aβ and STZ, but it does assist in increasing the anti-inflammatory effects of *C. cicadae* fermentation products.

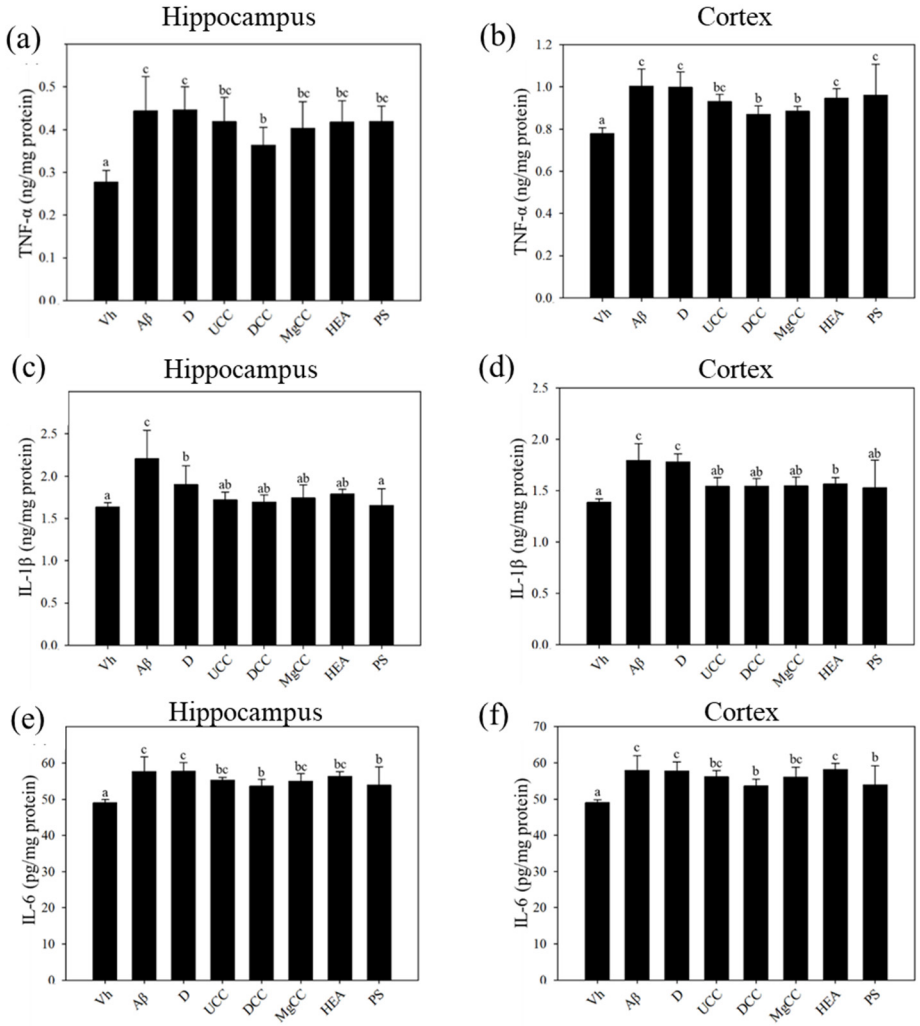

Figure 4. Effect of various *C. cicadae* NTTU 868 fermented products on TNF-α (**a**,**b**), IL-1β (**c**,**d**), and IL-6 (**e**,**f**) protein expression of rats with AD induced by Aβ40 and STZ i.c.v. infusion in the hippocampus and the cortex. Groups of rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

3.5. Effects of C. cicadae Fermentation Products on the Expression of sRAGE in the Hippocampus of Rats with Brain Infusions of Aβ40 and STZ

An anti-inflammatory factor, sRAGE when binding with RAGE reduces the binding of Aβ and RAGE and the production of proinflammatory factors [30,31]. As demonstrated in Figure 5, after Aβ40 and STZ infusions, the expression of sRAGE was significantly lower than in the Vh group ($p < 0.05$), and feeding DOW, UCC, DCC, and MgCC can effectively increase the expression of sRAGE, with DCC having the most significant increases ($p < 0.05$), followed by MgCC ($p < 0.05$). This result indicates that the sRAGE-enhancing effect of DCC may originate from synergistic effects of the Mg^{2+} in DOW and other ions after being involved in the cocultivation of *C. cicadae* to increase sRAGE expression and reduce inflammation responses.

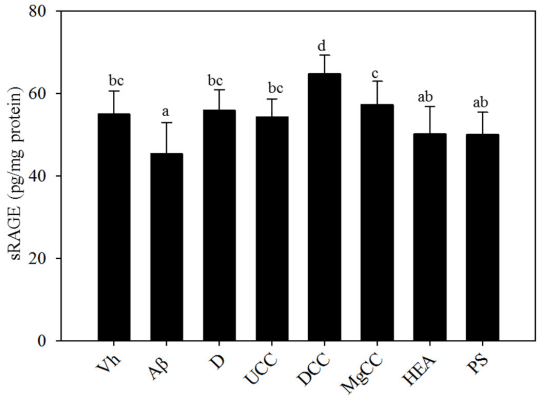

Figure 5. Effect of various *C. cicadae* NTTU 868 fermented products on sRAGE protein expression of rats with AD induced by Aβ40 and STZ i.c.v. infusion in the hippocampus. Rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c,d} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

3.6. Effects of C. cicadae Fermentation Products on Mg^{2+} in the Blood Serum, Hippocampus, and Cortex of Rats with Brain Infusions of Aβ40 and STZ

Studies have demonstrated Mg^{2+} imbalances in individuals with AD and lab animals with induced AD and that moderate supplementation of Mg^{2+} can effectively improve AD symptoms [32–35]. Therefore, moderate supplementation of Mg^{2+} is viewed as a key indicator in AD improvement. The results are presented in Table 2. Brain infusions of Aβ40 significantly reduced Mg^{2+} content in the blood serum, hippocampus, and cortex ($p < 0.05$). DCC significantly increases the concentration of Mg^{2+} in the hippocampus and the cortex by 16.0% and 16.0%, respectively ($p < 0.05$). MgCC also increases the concentration of Mg^{2+} in the hippocampus and the cortex by 9.7% ($p > 0.05$) and 11.2% ($p < 0.05$), respectively. This confirms that DCC can effectively treat Mg^{2+} imbalances in the brain.

Table 2. Effect of various *C. cicadae* NTTU 868 fermented products on Mg^{2+} concentration in the serum, hippocampus, and cortex of rats with AD induced by Aβ40 and STZ i.c.v. infusion.

Group	Mg^{2+} Concentration		
	Serum (mg/dL)	Hippocampus (μg/g)	Cortex (μg/g)
Vh	5.08 ± 0.44 ^{a}	2.24 ± 0.06 ^{c}	1.45 ± 0.19 ^{b}
Aβ	3.92 ± 0.50 ^{b}	1.75 ± 0.15 ^{a}	1.25 ± 0.05 ^{a}
D	4.10 ± 0.49 ^{b}	1.87 ± 0.16 ^{ab}	1.32 ± 0.07 ^{ab}
UCC	3.83 ± 0.66 ^{b}	1.87 ± 0.22 ^{ab}	1.33 ± 0.07 ^{ab}
DCC	4.02 ± 0.74 ^{b}	2.03 ± 0.18 ^{b}	1.45 ± 0.11 ^{b}
MgCC	3.73 ± 0.30 ^{b}	1.92 ± 0.14 ^{ab}	1.39 ± 0.08 ^{b}
HEA	3.90 ± 0.33 ^{b}	1.88 ± 0.09 ^{ab}	1.32 ± 0.12 ^{ab}
PS	3.87 ± 0.40 ^{b}	1.74 ± 0.11 ^{a}	1.25 ± 0.08 ^{a}

Groups of rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

3.7. Effects of C. cicadae Fermentation Products on the Expression of MAGTI Protein in the Hippocampus of Rats with Brain Infusions of Aβ40 and STZ

MAGT1 protein on cell membrane carries Mg^{2+} outside the membrane into the cell, increasing Mg^{2+} content inside the cell. Increased Mg^{2+} content in brain cells reduces the expression of BACE, thus reducing the Aβ generation caused by the cleavage of APP by BACE [32,33]. Related studies have also confirmed that Mg^{2+} in brain cells can reduce hyperphosphorylation of p-tau protein by inhibiting GSK-3β expression (Gomez et al., 2006). As in Figure 6, which illustrates MAGT1 in the hippocampus of rats with AD, because brain infusions of Aβ40 and STZ reduce Mg^{2+} in the brain, MAGT1 levels drop significantly ($p < 0.05$). UCC, HEA, and PS did not increase MAGT1 expression, whereas D, DCC, and MgCC effectively increased expression of MAGT1 ($p < 0.05$). This indicates that D, DCC, and MgCC of Mg^{2+} can reduce the generation of Aβ by regulating the expression of MAGT1.

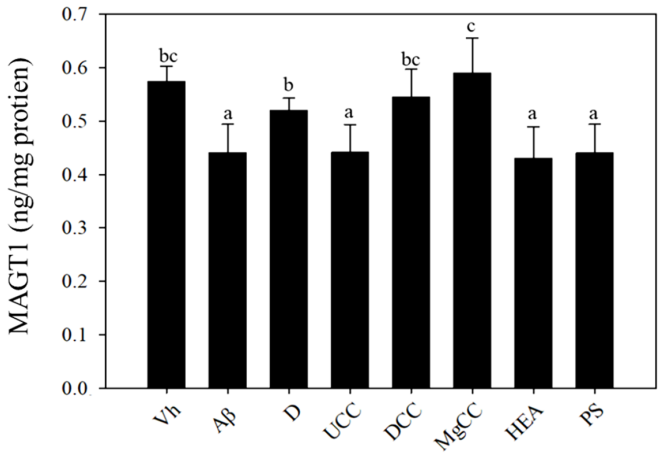

Figure 6. Effect of various *C. cicadae* NTTU 868 fermented products on MAGT1 protein expression of rats infused with Aβ40 and STZ in the hippocampus. Groups of rats were infused with vehicle solution (Vh group) or Aβ40 solution (Aβ group) by i.c.v. injection without administration of test materials. Other Aβ40- and STZ-infused rats were administered deep ocean water (0.0976 mL/kg/day, deep ocean water (DOW) group), ultra-pure water cultured *C. cicadae* (UCC) (200 mg/kg/day, UCC group), DOW-cultured *C. cicadae* (DCC) (220 mg/kg/day, DCC group), $MgCl_2$ solution-cultured *C. cicadae* (MgCC) (137 mg/kg/day, MgCC group), N6-(2-hydroxyethyl)-adenosine) (1.12 mg/kg/day, N(6)-(2-Hydroxyethyl) adenosine (HEA) group), or polysaccharides (1.5 mg/kg/day, PS group). The Mg^{2+} concentration of DCC and MgCC was 309.75 mg/L. Data are presented as mean ± SD; ^{a,b,c} Different letters indicate significantly different values according to a one-way ANOVA using Duncan's multiple test ($p < 0.05$).

4. Discussion

Studies have argued that DOW can promote the growth of fungal mycelium and regulate the production of functional components [15]. This study examined the cultivation of *C. cicadae* using UPW, DOW, and $MgCl_2$. Compared with using UPW, cultivating *C. cicadae* with DOW increased mycelium growth and HEA content but reduced intracellular and extracellular polysaccharide contents. Regarding the mycelium growth, *C. cicada* were similar to other fungi such as *Monascus purpureus*, *Antrodia cinnamomea*, and *Cordyceps militaris* can be significantly increased by DOW [12,14–16,36]. According to our past research, different minerals in DOW may affect the growth of *C. militaris* and the production of cordycepin [16]. $MgCl_2$ was regarded as the most important compound in the DOW. However, $MgCl_2$ and DOW performed the opposite trend on the regulation of mycelium production. $MgCl_2$ largely reduced the production of mycelium and adenosine, increased HEA content, and reduced the production of intracellular polysaccharides. The lower intracellular polysaccharides production may result from the decreased mycelium production. Although the polysaccharide content is reduced, it may also change the polysaccharide

composition. The crude polysaccharides isolated from DCC still have the ability to slightly improve memory deficit and anti-inflammatory effect. It shows that the polysaccharide composition of DCC has an improvement effect on the development of AD.

Past studies have pointed out that the Cl^- in DOW would reduce the production of cordycepin and mycelium. *C. militaris* mycelium was increased by $MgNO_3$ but not by $MgCl_2$ [16]. Therefore, in this study, the main factor that $MgCl_2$ reduces the production of *C. cicadae* mycelium and adenosine may not be caused by Mg^{2+}, but by Cl^-. It is the first study to prove that DOW enhanced mycelium and HEA productions of *C. cicadae*. Although the production of polysaccharides was slightly decreased ($p > 0.05$), the co-fermentation product still performed the synergistic functional effect. This study also found that the mycelium of *C. cicadae* can absorb Mg^{2+} in DOW and increase the content of Mg^{2+} in cells. Mg^{2+} was absorbed in cells and converted into organic ions, and the absorbed Mg^{2+} should be the important factor for increasing magnesium uptake in brain (Table 2).

Aβ40 is a neurotoxic protein generated by cleaved APP and leads to immense production of inflammatory factors that cause damage to nerve cells and promote excessive phosphorylation of tau proteins. As a result, neurofibrillary tangles continually form and block the transmission of neural signals, which inhibits memory [26,27]. STZ causes necrosis of pancreatic β cells, affecting the secretion of insulin and resulting in DM; as a result, it has often been considered a drug that induces type 1 diabetes. STZ promotes the accumulation of Aβ in the brain and causes the excessive phosphorylation of tau proteins; this damages nerve cells and blocks the transmission of neural signals, affecting learning and memory. In recent years, it has been considered a drug that induces AD [37]. In this study, Aβ40 and STZ were used as drugs to co-induce AD; a mixture of Aβ40 and STZ was infused into the brains of animals for 28 days to induce AD. The results demonstrate that infusions of Aβ40 and STZ effectively impede the learning and memory of rats. An analysis of AD risk factors and inflammatory factors indicated that infusions of a Aβ40 and STZ mixture effectively increased the expression of AD risk factors Aβ, BACE, and p-tau, decreased the expression of microglia cell receptor sRAGE, and increased the expression of inflammatory factor TNF-α, IL-1β, and IL-6 proteins. This then increases the expression of BACE and causes the continuous synthesis of Aβ, leading to hyperphosphorylation of tau proteins and massive formation of neural tangles and senile plaques. This ultimately affects memory and learning, inducing AD.

The water maze involves the observation of rodents searching for a hidden platform submerged in a pool of water and recording the time and swimming trajectories of the animals locating the platform. The records are then analyzed to deduce memory and spatial awareness. The reference memory laboratory placed a hidden platform in quadrant IV and recorded the time the rats spent searching for the platform each instance; this was one of the methods employed to evaluate their learning and memory. Injections of Aβ40 and STZ into the brain reduced memory learning in the reference memory test, probe test, and working memory test. UCC had poor results in improving memory and learning, but DCC and MgCC improved memory and learning considerably, with DCC outperforming MgCC. This result may be related to the main functional components; DOW, HEA, and polysaccharides are the main functional components in this study. In the probe test, polysaccharides exhibited more favorable results in improving memory and learning than did HEA. However, although UCC had the highest polysaccharide content, it did not demonstrate significant effectiveness (Table 1). Therefore, the reason for DCC exhibiting more satisfactory results in improving memory and learning is not its higher HEA and polysaccharide content. Furthermore, MgCC also led to significant improvements, indicating that the ions in DOW may be a main factor driving DCC improvement of memory and learning capabilities. The effectiveness of DCC may be due to increases in polysaccharide and HEA content in *C. cicadae* (for HEA after DOW cultivation) and the content of Mg and other minerals in fermented *C. cicadae* mycelium. UCC contains only polysaccharides and HEA; therefore, its effects are limited.

Aβ is generated after the cleavage of APP by BACE and γ-secretase [23]. A result of increased BACE expression or enzyme activity in the brain is the generation of Aβ. Therefore, the expression of Aβ, BACE, and p-tau proteins were measured in this study to determine whether the effects of DCC in improving memory and spatial learning derive from regulating the formation of Aβ and the phosphorylation of tau proteins. After inducements with Aβ and STZ, the expression of Aβ and BACE was increased significantly ($p < 0.05$). DCC can significantly reduce the expression of Aβ and BACE when their levels have been elevated, whereas UCC, MgCC, HEA, and PS can slightly lower Aβ and BACE expression. This demonstrates that DCC can mitigate memory loss by regulating Aβ-generation pathways, and this effect may be due to ions in DOW and the synergistic effects of HEA and PS in *C. cicadae*. Phosphorylation of tau proteins is then aggravated; hyperphosphorylation of tau proteins in the brain diminishes microtubal organization and results in the formation of neural tangles. This impairs the transmission of nerve substances, resulting in some neuronal components that are typically transported between cell bodies and nerve endings (and are not rapidly degraded) accumulating between normal neuronal cells. This ultimately leads to degeneration of the neuronal cells [24,26,27]. Among the experimental substances, only MgCC significantly reduced p-tau protein expression ($p < 0.05$); no significant differences in p-tau protein expression were present between the other experimental substance groups and the Aβ group.

When Aβ is produced in massive quantities and accumulated in brain cells, the excessive Aβ binds with RAGE receptors on microglia cells, which increases the expression of inflammatory factors such as TNF-α, IL-1β, and IL-6 and induces massive release of inflammatory factors. This leads to continuous inflammatory responses and results in the apoptosis of many nerve cells [30]. The results of this study indicate that DCC can significantly reduce ($p < 0.05$) the expression of TNF-α, IL-1β, and IL-6, elevated by infusions of Aβ40 and STZ, in the hippocampus and the cortex.

As a protein that regulates RAGE, when sRAGE is synthesized with RAGE, it blocks the synthesis of intracellular Aβ and RAGE. This suppresses the production of pro-inflammatory factors and reduces the occurrence of inflammatory responses [31,38]. Feeding DCC can increase the expression of sRAGE. Among its main components, HEA and PS also slightly increase the expression of sRAGE. These results indicate that HEA and PS in DCC increase expressions of sRAGE and reduce the expression of pro-inflammatory factors TNF-α, IL-1β, and IL-6; this then reduces the production of inflammatory responses. These effects may be due to the abundant minerals in DOW as well as the Mg^{2+} fermentation product of the co-fermentation of DOW and *C. cicadae* and the effects of HEA and PS in *C. cicadae*.

According to past research, although *Cordyceps cicadae*, *C. Militaris*, and *C. sinensis* belong to *Cordyceps* species, the functional components produced are quite different. In additoin to the fungi polysaccharides, *C. militaris* is a famous for higher production of cordycepin. Cordycepin in *Cordyceps militaris* has been considered as one of the functional components with neuroprotection in past studies [39]. *Cordyceps cicadae* has been gradually discovered in recent years because its HEA was proven to perform liver protection [4], renoprotection [2], and anti-diabetic effect [39,40]. Some studies have argued that HEA in *C. cicadae* can limit NF-κB signal pathways and alleviate LPS-induced inflammatory responses [1]. *C. cicadae* polysaccharides were also proven to perform anti-oxidation and anti-inflammatory activities [2]. In AD prevention, anti-inflammatory should be an important feature against Aβ-induced neurodamage. In this study, it was found that HEA and polysaccharides of *Cordyceps cicadae* exhibit neuroprotection effects due to their anti-inflammatory effects. In the analysis results of cortex and hippocampal tissue, HEA reduced Aβ-induced IL-1β expression, and polysaccharides also significantly repressed IL1β and IL-6. Therefore, HEA and *C. cicadae* polysaccharides still performed some potentials on suppressing inflammatory responses in AD brain.

In addition, *Cordyceps militaris* and *Cordyceps cicada* have similar effects on DOW fermentation. DOW can improve both of the biomass and health benefits. However, in

particular, *Cordyceps cicada* can convert DOW minerals into a form that is beneficial to biological absorption. In this study, organic Mg^{2+} of DCC was the important key factor to achieve neuroprotective effects. Mg^{2+} is among the most abundant bivalent cations in cells. It synthesizes with enzymes and related receptors in cells to regulate cell growth, metabolism, and neurotransmissions. Past studies have demonstrated deficiencies in brain magnesium content in AD animal models and feeding test subjects with experimental substances containing magnesium can effectively supplement Mg^{2+} deficiencies in the brain and improve AD symptoms [35]. Therefore, using magnesium content in the hippocampus and cortex as the study basis, our experiments investigated whether feeding the subjects with experimental substances containing magnesium can increase magnesium content in the brain and thus suggest the possibility of treating AD. Rats infused with a mixture of Aβ40 and STZ had significantly lower magnesium content in their hippocampus and cortex than rats undergoing vesicle. Being fed DCC and MgCC effectively supplemented Mg^{2+} content in the hippocampus and the cortex. Feeding DOW alone only slightly increased the Mg^{2+} content in the hippocampus and the cortex. Organic Mg^{2+} effectively mitigated the Mg^{2+} deficiency in the brain. Feeding the DOW and Mg^{2+} fermentation products in *C. cicadae* yielded considerably more satisfactory results, which can be attributed to the co-fermentation of DOW and $MgCl_2$ with *C. cicadae* producing organic state Mg^{2+} products that are more conducive to absorption and use.

MAGT1 in the brain facilitates Mg^{2+} entering neuronal cells to suppress the expression of BACE, reduces the formation of Aβ, and inhibits the expression of GSK-3β. This reduces the phosphorylation of tau proteins [34]. The results of our experiment indicated that rats infused with a mixture of Aβ40 and STZ have significantly lower expression of MAGT1 than rats undergoing sham operations ($p < 0.05$). This finding is consistent with those of past studies in which mice with induced AD had significantly lower expression of MAGT1, and their being fed experimental substances with Mg^{2+} significantly increased MAGT1 expression ($p < 0.05$); MgCC exhibited the greatest increase [32,33]. However, in our study, feeding HEA and PS did not result in significant differences ($p > 0.05$), indicating that the outcomes from DCC and MgCC were not due to the effects of HEA and PS but were possibly due to the Mg^{2+} in the DOW and $MgCl_2$ mixture. MgCC contains only single-ion magnesium, which may be more easily used by *C. cicadae* to increase the expression of MAGT1. DCC, possibly because it is restricted by other ions in DOW and its role in maintaining ion consistency in the body, resulted in lower expression of MAGT1 than that in rats fed with MgCC. These observations suggest that after DOW and $MgCl_2$ were co-fermented with *C. cicadae*, the resulting magnesium fermentation products assist with replenishing magnesium deficiency in the brain and are also conducive to increasing the expression of MAGT1 in the brain and improving AD.

UCC, DCC, and MgCC can reduce the synthesis of Aβ and RAGE by increasing the expression of sRAGE. This results in the deactivation of a large quantity of microglia cells, which lowers the expression of TNF-α, IL-1β, and IL-6 and reduces the occurrence of inflammatory responses. Due to co-fermentation with DOW and $MgCl_2$ to become fermentation products containing magnesium, DCC and MgCC can effectively regulate MAGT1. Summarizing these effects, UCC only improved inflammatory responses, and this effect is due to the functional components HEA and *C. cicadae* polysaccharides already present in *C. cicadae*. MgCC contains the inflammatory abilities of HEA and *C. cicadae* polysaccharides, but it can also regulate MAGT1 expression due to its fermentation with $MgCl_2$ producing fermentation products containing organic magnesium. As a result, compared with UCC, MgCC has more potential to improve AD. DCC contains *C. cicadae* HEA and *C. cicadae* polysaccharides and has the ability to improve inflammatory responses; it also contains organic fermentation products with DOW minerals and magnesium, enabling effective MAGT1 regulation. Combined with the synergistic effects provided by its other ions, DOW outperforms MgCC and UCC in treating AD.

This study investigated the effects of *C. cicadae* fermentation products on absorbing ions and treating AD induced by Aβ 1–40 and STZ. *C. cicadae* absorbs Mg^{2+} in DOW at

a high level, which increases its growth and the production of its active components as well as the ion-absorption rate of organisms. The mechanisms were shown in Figure 7. Studies on models of animals with AD have demonstrated that *C. cicadae* cultivated with DOW contains the anti-inflammatory substances HEA and *C. cicadae* polysaccharides. Furthermore, due to effects from its co-cultivation with DOW, *C. cicadae* can increase the expression of MAGT1 and increase Mg^{2+} content in the cortex as well as effectively increase the expression of sRAGE and inhibit the release of inflammatory factors by microglia cells. This effectively reduces the occurrence of inflammatory responses, which lowers the expression of BACE and reduces the generation of Aβ. In summary, *C. cicadae* cultivated with DOW can treat AD through the effective supplementation of Mg^{2+} required by the human body and the inhibition of inflammatory responses.

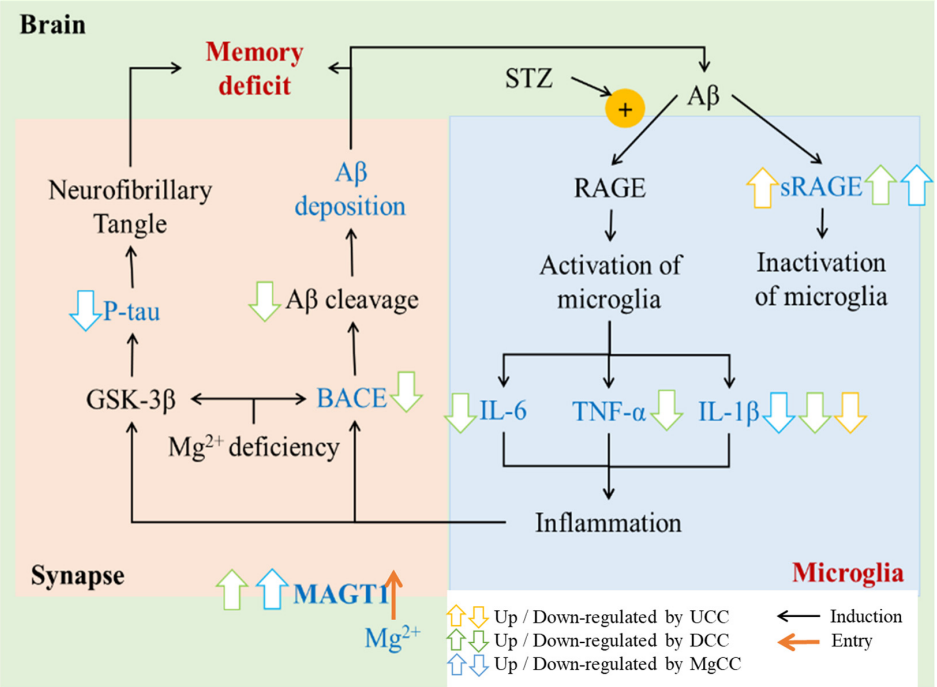

Figure 7. Mechanisms of various *C. cicadae* NTTU 868 fermented products in the regulation of the formation of Aβ and pro-inflammatory response in STZ- and Aβ40-induced Alzheimer's disease.

Author Contributions: Y.-Z.W.: experiments on fermentation, functional compound analysis, and animal test; C.-L.L.: experiments, experiment design, funding application, experiment discussion, paper writing, and submission. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taitung University (protocol code 1050321 and 2016/03/21 of approval).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data included in this study are available upon request by contacting the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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