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Docosahexaenoic Acid (DHA), Vitamin D3, and Probiotics Supplementation Improve Memory, Glial Reactivity, and Oxidative Stress Biomarkers in an Aluminum-Induced Cognitive Impairment Rat Model

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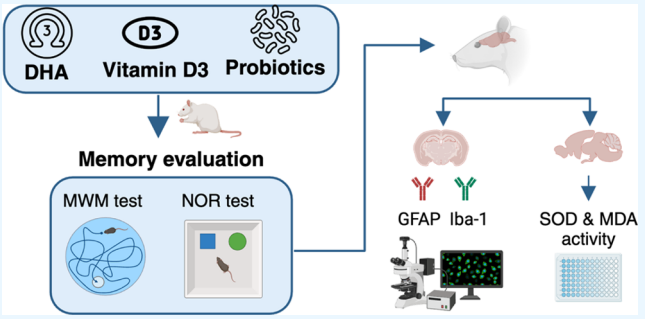
ABSTRACT: Globally, the rise in neurodegenerative issues in tandem with shifts in lifestyle and aging population has prompted a search for effective interventions. Nutraceutical compounds have emerged as promising agents for addressing these challenges. This 60-day study on an aluminum-induced cognitive impairment rat model assessed three compounds and their combinations: probiotics (Prob, *Lactobacillus plantarum* [5×10^{10} CFU/day], and *Lactobacillus acidophilus* [5×10^{10} CFU/day]), docosahexaenoic acid (DHA, 23.8 mg/day), and vitamin D3 (VD3, 150 IU/day). Behavioral outcomes were evaluated by using the Morris water maze and novel object recognition tests. Glial activation was assessed through immunofluorescence analysis of GFAP/Iba1, and oxidative stress markers in brain tissue were determined by measuring the levels of Malondialdehyde (MDA) and Superoxide dismutase (SOD). The results demonstrated a progressive improvement in the learning and memory capacity. The aluminum group exhibited the poorest performance in the behavioral test, enhanced GFAP/Iba1 activation, and elevated levels of oxidative stress markers. Conversely, the DHA + Prob + VD3 treatment demonstrated the best performance in the Morris water maze. The combination of DHA + Prob + VD3 exhibited superior performance in the Morris water maze, accompanied by reduced levels of GFAP/Iba1 activation in DG/CA1 brain regions. Furthermore, DHA + Prob supplementation showed lower GFAP/Iba1 activation in the CA3 region and enhanced antioxidant activity. In summary, supplementing various nutraceutical combinations, including DHA, VD3, and Prob, displayed notable benefits against aluminum-induced cognitive impairment. These benefits encompassed memory enhancement, diminished MDA concentration, increased SOD activity, and reduced glial activation, as indicated by GFAP/Iba1 markers.



1. INTRODUCTION

The human brain, a central hub orchestrating movement, emotions, and memory, underscores the imperative of maintaining its health.[1] Neurodegenerative diseases, impacting professional, familial, and social realms, necessitate attention due to their medical and socioeconomic repercussions, affecting individuals' professional, familial, and social dimensions by impeding daily activities. Manifestations include motor and cognitive impairments, memory loss, dementia, and respiratory difficulties, among others. The diagnosis often occurs at advanced stages, limiting the efficacy of treatments.[2]

Worldwide, the rise in brain-related issues correlates with lifestyle changes and the aging population. High-risk factors such as stress, obesity, sedentary behavior, and poor nutrition contribute substantially to the increased incidence of these neurological conditions.[3] Furthermore, intrinsic factors like aging, combined with lifestyle choices, exacerbate neurodegeneration and cognitive impairment. Conversely, adopting a healthy lifestyle has been associated with a reduced risk of developing such conditions, emphasizing the potential of specific dietary components as anti-inflammatory, antioxidant, or protective agents.[3,4]

Recognizing the pivotal role of diet in physical and mental well-being, researchers focus on brain preservation through a healthy diet.[5] The food industry is responding with next-generation functional foods, incorporating bioactive ingredients to combat chronic degenerative diseases and provide

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Table 1. Composition of Experimental Treatments for Rats

treatment	water	tween 80[a]	Al[a]	DHA[a]	VD3[a]	Prob[a]
control (−)[a]	x	x				
Al (+)	x	x	x			
Al + DHA	x	x	x	x		
Al + VD3	x	x	x		x	
Al + Prob	x	x	x			x
Al + DHA + VD3	x	x	x	x	x	
Al + DHA + Prob	x	x	x	x		x
Al + Prob + VD3	x	x	x		x	x
Al + DHA + VD3 + Prob	x	x	x	x	x	x

[a]Tween 80 (5%); Control = healthy individuals; Al = aluminum chloride (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5 × 10^{10} CFU/day], *L. acidophilus* [5 × 10^{10} CFU/day]). Each treatment was administered with purified water and fed with a conventional diet.

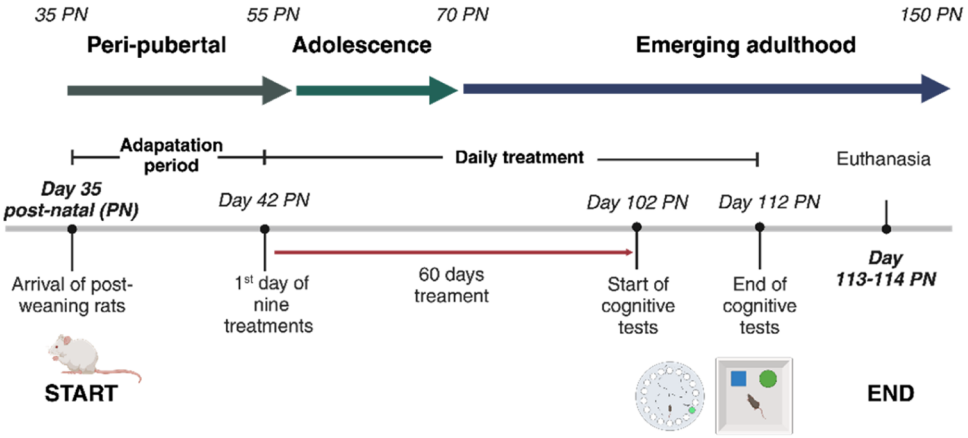

Figure 1. Experimental protocol summary of the treatments administration. DHA, Vitamin D3 (VD3), and probiotics (Prob) were coadministered with aluminum chloride (Al) during the 60-day experimental protocol considering a preventative scheme for cognitive impairment (Created with BioRender.com).

benefits beyond mere nutrition.[6] In this context, it has been found that nutraceuticals can modulate brain functions such as memory, cognition, neuroprotection, neuro-regeneration, and neuroplasticity.[7,8] Clinical evidence shows that nutraceuticals, such as omega-3 fatty acids (DHA), vitamin D3 (VD3), and probiotics, exhibit promising effects in preventing or ameliorating neurodegenerative diseases.[9−13]

Studies have verified that the adequate consumption of DHA has protective properties, showcasing its ability to limit synaptic loss, cognition defects, and even suppress neural damage promoted by amyloid β and hyperphosphorylated tau accumulation in Alzheimer's disease models.[14−16] Vitamin D has shown neuroprotective potential in several studies through its involvement in antioxidant pathways, neurotrophin production, neuronal calcium and glutamate regulation, immunomodulation, and amyloid β clearance.[17−19] Probiotics, influencing the gut-brain axis, are emerging as biotherapies for neurodegenerative diseases, positively impacting neurotrophic factors and reducing oxidative stress, influencing neuronal plasticity.[10,20−27]

While data suggests that nutrition and exercise can reduce the risk of neurodegenerative disease development, the combined effects of probiotics, multivitamins, and omega-3 may enhance brain performance, thus attenuating symptoms associated with neurological disorders.[28] Despite individual benefits being established, the synergistic impact of these compounds remains unexplored. The present study evaluates the impact of DHA, VD3, and probiotics (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) individually and combined in an aluminum chloride (Al)-induced cognitive impairment rat model, aiming to unveil their effects on cognitive abilities, brain inflammation, and oxidative stress.

2. MATERIALS AND METHODS

2.1. Reagents and Active Ingredients. DHA (life's DHA S17−P100) and VD3 (dry vitamin D3 100 SD/S) were provided by DSM (Heerlen, NL). Probiotic strains (Prob, *L. plantarum*, and *L. acidophilus*) were purchased from ENZIQUIM (CDMX, MX). Aluminum chloride ($AlCl_3$) was purchased from Karal (GTO, MX).

2.2. Animals. Male Wistar rats (N = 74) of 35 postnatal (PN) days were obtained from Bioterio Morelos (MOR, MX) and housed in translucent polycarbonate cages at room temperature (25 ± 2 °C) and 12/12 h light-dark cycle. Access to water and food was ad libitum. All experimental manipulations were carried out following international ethical standards based on the Guide for Care and Use of Laboratory Animals and national standards based on NOM-062-ZOO-1999, and the protocol was submitted for evaluation by the Internal Committee for the Care and Use of Laboratory Animals (Code: 2022−015A).

2.3. Diet Administration. Rats were administered from their periadolescence (42 PN) phase through early adulthood (112 PN) to evaluate the use of these compounds as preventive agents for neurological diseases. The aging of the population is a critical factor in developing chronic neuro-

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logical diseases; thus, prevention is an important goal to preserve the quality of life and to extend a healthy life span.[29] After 1 week of acclimatization, on the 42 PN day, animals were randomly distributed into the experimental groups. A total of nine treatments were evaluated as indicated in Table 1: (1) control [n = 9], (2) aluminum chloride control (Al) [n = 9], (3) DHA [n = 8], (4) VD3 [n = 8], (5) Prob [n = 8], (6) DHA + VD3 [n = 8], (7) DHA + Prob [n = 8], (8) Prob + VD3 [n = 8], and (9) DHA + VD3 + Prob [n = 8].

Figure 1 summarizes the experimental protocol. The induction of cognitive impairment in rats was carried out by oral gavage administration of 100 mg/kg of Al (aluminum) over a 60-day period, in concordance with methodologies previously reported in other studies.[18−21] Likewise, all treatments were administered daily for 60 days using oral gavage. Al was administered at least two h prior to the treatment to ensure the stability of bioactive compounds, particularly the viability of probiotics. All treatments were prepared daily. Experiments were conducted during the light phase, spanning from 09:00 to 17:00 h. The weight of the animals was monitored once a week throughout the study.

2.4. Memory Evaluation. After the 60-day treatment period, behavioral tests were carried out to establish learning and memory, according to Reza-Zaldivar *et al.*[30] Supplementation of the treatments was continued during these tests.

The Morris water maze (MWM) consisted of a round pool of 150 cm in diameter and 50 cm in depth, with a black background, and filled with water (25 ± 2 °C). The pool was virtually divided into four quadrants, and spatial cues were placed around the pool and remained unchanged throughout the experiment. These spatial cues were used to support the animals' reference memory.[18] In addition, a small transparent square platform of 10 cm × 10 cm was placed at 1 cm under water level in one of the quadrants. The rats received a training phase consisting of daily sessions for five consecutive days. The animal was positioned within the pool in one of the quadrants, facing the wall of the pool, and allowing them to swim freely for 60 s until they reach the platform. Animals unable to reach the platform were guided and placed on it for 20 s; then they had a rest period of 15 min prior to repeating the trial. The drop location was changed for each trial. The entire process was videotaped for analysis. The parameters to evaluate in this test were the swimming time and distance to reach the platform.[30]

The novel object recognition (NOR) test consisted of three phases: the habituation phase, in which 24 h before the test, the animal was placed in an exploration field for 5 min to become familiar with the environment. The familiarization phase in which the animals were placed for 5 min in the same exploration field in the presence of two identical objects, followed by the animals returning to their cages for 15 min, and finally, the testing phase, in which the animals were placed back on the exploration field for 5 min; however, one of the two objects were exchanged for a different one. During each change of animal and object, cleaning with 70% ethanol was done to avoid recognizing odors.[30] The discrimination index (DI) was determined with the following formula:

$$\mathrm{DI} = \frac{\text{time spent with the novel object}}{\text{time spent with the both object}} \times 100$$

All of the behavioral test parameters were evaluated and analyzed with the ANY-maze 5.26 video tracking system (IL, 2017).

2.5. Brain Obtention. Animals were anesthetized with sodium pentobarbital (150 mg/kg). Furthermore, rats were euthanized using a direct pericardial puncture. Next, the brains were removed and cut in half. One-half of the brains were stored at −80 °C, and the other was fixed by immersion in 4% paraformaldehyde at pH 7.4. Fixed brains were subjected to a cryoprotection treatment by immersion in 10, 20, and 30% sucrose with 0.1% sodium azide. Afterward, brains were embedded in a tissue-freezing medium (Leica Biosystems, Wetzlar, GER) and frozen in a cryostat (CM1850, Leica Biosystems, Wetzlar, GER). Cryopreserved brains were cut coronally into sections of 10−20 μm thick. The anatomical section evaluated was the hippocampus, which is one of the main areas associated with memory.

2.6. Immunofluorescence of the Brains. The effect of the treatments on glial activation was determined using the GFAP and Iba1 antibodies. For immunofluorescence, coronal sections were obtained from the nine treatments. The blocking was performed with 2.5% horse serum and 0.5% Triton-PBS for 1 h. Subsequently, the coronal sections were incubated with mouse anti-GFAP 4648 (1:100; Abcam, Cambridge, MA) and rabbit anti-Iba1 178846 (1:500; Abcam, Cambridge, MA) overnight at 4 °C. Later, sections were incubated at room temperature for 2 h using secondary antibodies Alexa 594 antimouse A11005 (1:1000; Thermo Fisher Scientific, MA) and Alexa 488 antirabbit A32731 (1:1000; Thermo Fisher Scientific, MA) protected from light. Sections were observed by using a fluorescence LED microscope (DM4 B, Leica Microsystems, Wetzlar, GER). Images were taken using Leica Application Suite (LAS) X version 3.1.1.15751 software and camera DFC7000T color CCD (Leica Microsystems, Wetzlar, GER) with a 40× objective. The quantitative analysis was performed on the anatomical areas of hippocampus CA1, CA3, and dentate gyrus (DG). The positive cells were counted according to standard methods[31] in a blinded fashion.

2.7. Oxidative Stress Markers. Brain samples stored at −80 °C were used to analyze the lipid peroxide malondialdehyde (MDA) and superoxide dismutase (SOD) activity. To analyze these parameters, lipid peroxidation (MDA) assay (ab118970, Cambridge, U.K.) and superoxide dismutase activity assay (ab65354, Cambridge, U.K.) colorimetric kits were employed. Samples were homogenized with a buffer Lysis provided by the kits in a porcelain mortar and meticulously prepared following the procedures outlined in each kit. The concentrations of MDA were determined by referencing a standard curve. Absorbance was measured at MDA 695 nm (MDA) and 450 nm (SOD) using a Varioskan LUX microplate reader (Thermo Fisher Scientific, MA).

2.8. Statistical Analysis. Data obtained from the behavioral tests, the number of GFAP/Iba1-positive cells, and oxidative stress markers were analyzed using a one-way analysis of variance followed by an LSD test ($p < 0.05$). Statistical analyses were performed using JMP statistical software v17.2.0 (SAS Institute Inc., Cart, NC). Differences between groups were considered statistically significant when $p \leq 0.05$; results were presented as the mean ± SE.

3. RESULTS

3.1. Behavioral Tests. The study assessed the effects of VD3, probiotics, and DHA, both individually and in

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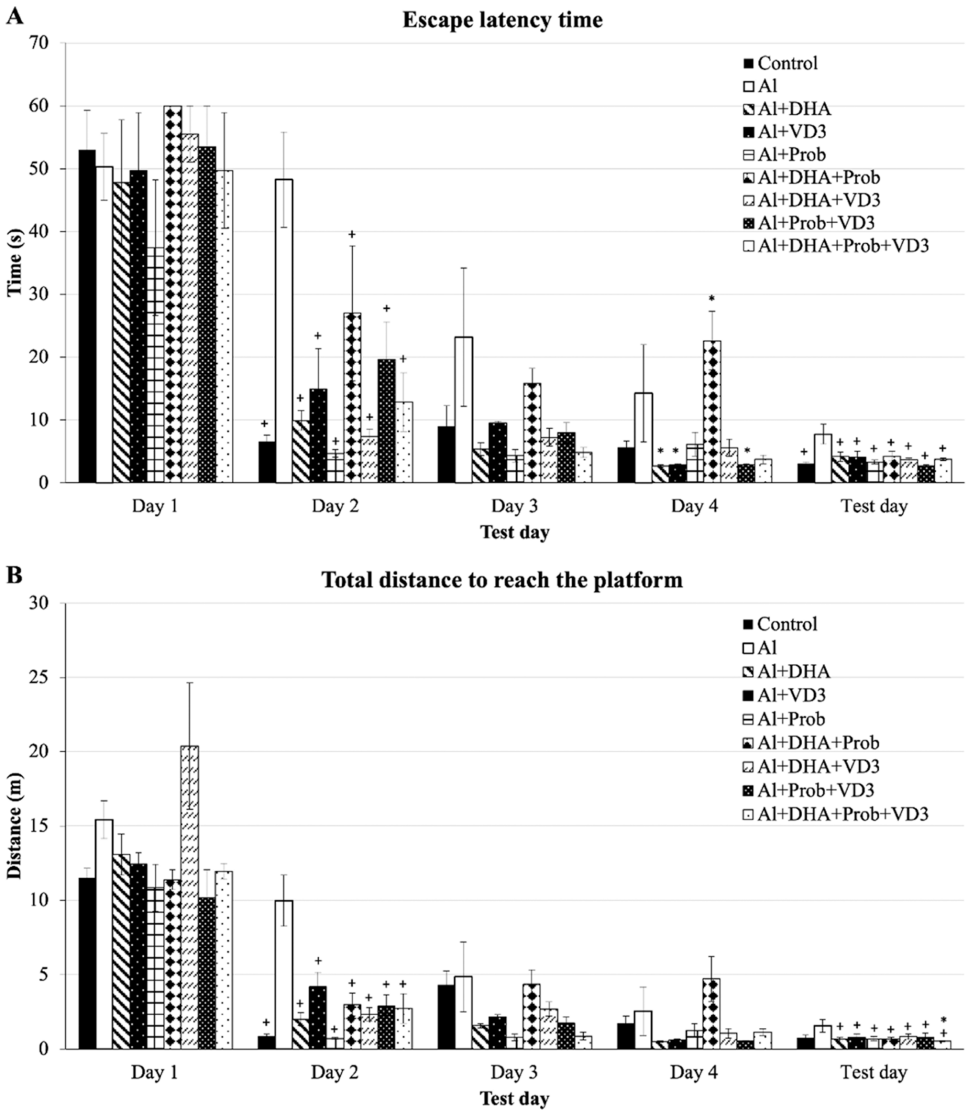

Figure 2. Morris water maze (MWM) test. (A) latency of rats from quadrant 2; (B) total distance traveled to reach the platform from quadrant 2. Control = healthy individuals; Al = aluminum chloride (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]). Asterisks (*) indicate a significant difference by the LSD test ($p < 0.05$) compared to the control, and crosses (+) indicate a significant difference by the LSD test ($p < 0.05$) compared to the Al control.

combinations, in a rat model with cognitive impairment induced by aluminum chloride. Aluminum exposure is known to induce neuronal degeneration, particularly in the cholinergic system, leading to clinical and pathological characteristics resembling those observed in neurological diseases such as Alzheimer's disease.[32] Here, MWM and NOR tests were performed to assess the cognitive capacity after Al and treatments administration. The results indicate that the administration of various treatments had an impact on the parameters assessed in both behavioral tests. Figure 2 illustrates changes in the MWM, where both the latency time (Figure 2A) and the swimming distance (Figure 2B) decreased over the training days.

During the second training session, all treatments exhibited a significant decrease ($p < 0.05$) in latency time compared to the Al treatment. By the third day, the Al + Prob + VD3 treatment showed a significant increase ($p < 0.05$) compared to the control. Animals treated with Al + DHA, Al + Prob + VD3, and Al + DHA + Prob + VD3 displayed lower swimming times from day 3 to day 4. On test day (session 5), animals treated with Al + Prob + VD3, Al + Prob, and Al + DHA + Prob + VD3 demonstrated significantly lower times, with decreases of 65.4, 57.36, and 51.68%, respectively, compared to the Al group. Similar trends were observed in the swimming distance (Figure 2B). During the second session day, all treatments showed a significant decrease ($p < 0.05$) in swimming distance compared to Al, except for Al + DHA + Prob. On test day, treatments Al + Prob + VD3, Al + Prob, and Al + DHA + Prob + VD3 exhibited significant decreases of 49.32, 56.2, and 65.86% in distance, respectively, compared to the Al control.

It is noteworthy that in both time and distance parameters, the Al treatment demonstrated poorer test performance than the control across all five sessions. On test day, all treatments significantly reduced the latency time. Al + Prob + VD3 showed the lowest latency time among all treatments, while Al + DHA + Prob + VD3 treatment not only displayed a more substantial percentage reduction compared to the Al group but

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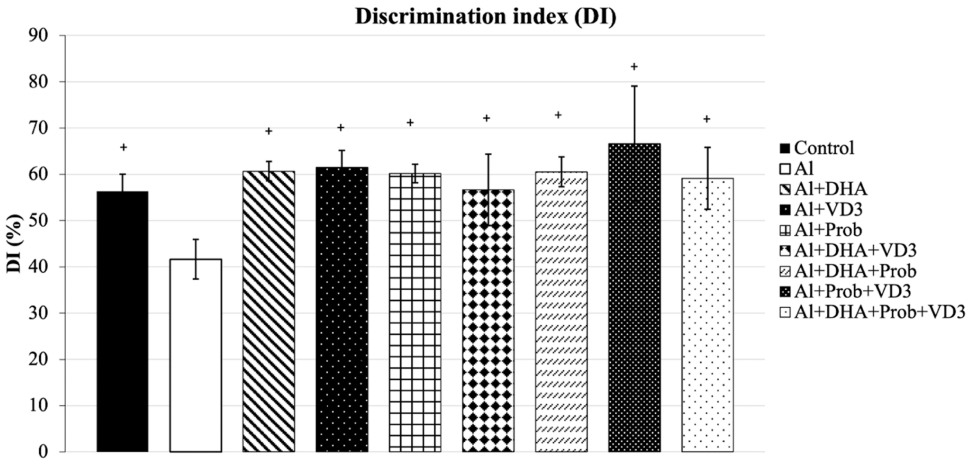

Figure 3. Novel object recognition test (NOR). Control = healthy individuals; Al = aluminum chloride control (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]). Asterisks (*) indicate a significant difference by the LSD test ($p < 0.05$) compared to the control, and crosses (+) indicate a significant difference by the LSD test ($p < 0.05$) compared to the Al control.

also exhibited a decrease in swimming distance by 30.82% compared to the control group.

Figure 3 presents the results of the NOR test. The data reveals a significant 26.1% decrease ($p < 0.05$) in the DI% for the Al group (41.64%) compared to the control group (56.32%), indicating reduced interaction of the rats with the novel object. In contrast, all treatments supplemented with bioactive compounds significantly increased ($p < 0.05$) the DI % compared to Al. Among the treatments, the Al + Prob + VD3 group demonstrated the most favorable NOR performance, showing a 59.9% increase in DI compared to the Al group. Notably, increases in DI% were observed for Al + DHA (7.7%), Al + VD3 (9.12%), Al + Prob (6.85%), Al + DHA + VD3 (0.6%), Al + DHA + Prob (7.5%), Al + Prob + VD3 (18.30%), and Al + DHA + Prob + VD3 (5%) treatments compared to the healthy control.

3.2. Immunofluorescence. Figure 4 displays the expression of GFAP and Iba1 in the dentate gyrus (DG) of the brains subjected to the negative stimulus (Al) and the various treatments evaluated in this study (Figures S1 and S2).

The results of the immunofluorescence analysis depicting GFAP and Iba-1 activation in three hippocampal areas (DG, CA1, and CA3) are illustrated in Figures 5 and 6. In Figure 5A−C, positive GFAP cells increased in the Al treatment compared to the control. In contrast, all treatments with bioactive compounds decreased cell activation compared to Al. Although no significant differences were observed between the treatments, a trend suggested a reduction in GFAP-positive cells in all three hippocampal regions with the combination of nutraceuticals. Remarkably, in the DG area, the Al + Prob + VD3 and Al + DHA + Prob + VD3 treatments exhibited a 41.8 and 50.9% reduction in GFAP-positive cells, respectively, compared to the Al group; in CA1, the same treatments reduced the GFAP-positive cells in 54.1% compared to the Al group ($p < 0.05$). Finally, in CA3, both treatments reduced the GFAP-positive cells by 28.57 and 33.33%, respectively.

Similar to the GFAP expression findings, Figure 6A−C illustrates an increase in microglia Iba1-positive cells in the Al treatment compared to the control in all hippocampal areas evaluated. However, a decrease in cell reactivity was observed for all treatments and across all hippocampal areas compared to that of the Al control. In Figure 6A, a significant decrease ($p < 0.05$) in the number of reactive cells compared to both the healthy (22.2, 27.7, 44.44, and 48.14%) and Al (36.36, 40.90, 54.54, and 57.57%) groups is observed for Al + DHA + Prob, Al + DHA + VD3, Al + Prob + VD3, and Al + DHA + Prob + VD3, respectively. Additionally, in the CA1 subfield (Figure 6B), the most significant decrease compared to the Al control is observed in Al + DHA + Prob + VD3 (44.44%), followed by Al + Prob (38.8%) and Al + DHA + VD3 (38.8%) treatments. Finally, for the CA3 subfield, the most significant decrease ($p < 0.05$) in the reactivity of microglial cells is shown for Al + DHA + Prob (42.85%), followed by Al + DHA + Prob + VD3 (35.71%) and Al + Prob + VD3 (28.57%) treatments compared to the Al control (Figure 6C).

3.3. Oxidative Stress Markers. MDA activity from brain samples is depicted in Figure 7A. Compared to the control, an increase in MDA concentration was observed for both Al and the treatments supplemented with bioactive compounds. Al administration led to a 411% increase in MDA production. Conversely, a significant decrease in MDA concentration was observed for all treatments except Al + DHA + Prob + VD3 compared to the Al control. Animals treated with Al + DHA + Prob, Al + DHA + VD3, Al + VD3, and Al + Prob + VD3 showed the lowest significant decreases ($p < 0.01$) of 62.85, 52.1, 54, and 45.19%, respectively, compared to the Al control. Furthermore, the SOD activity is illustrated in Figure 7B. A decrease in SOD activity of 57.60% is observed for the Al group compared to the control. The most significant increase ($p < 0.05$) in SOD activity was achieved with the Al + DHA + Prob treatment (217.13%) compared to the Al control, followed by Al + DHA + VD3, Al + Prob + VD3, and Al + VD3 with increases of 184.46, 167.33, and 163.34%, respectively. Additionally, Al + DHA + Prob, Al + DHA + VD3, and Al + Prob + VD3 showed a 34.45, 20.6, and 13.34% increase in SOD activity, respectively, compared to the healthy control.

4. DISCUSSION

4.1. Behavioral Tests. Aging involves inevitable neurodegenerative processes affecting brain functions and serves as a primary risk factor for chronic neurodegenerative diseases.[33] Despite the lack of a cure for age-related conditions, a cost-effective and easily administered nutritional approach

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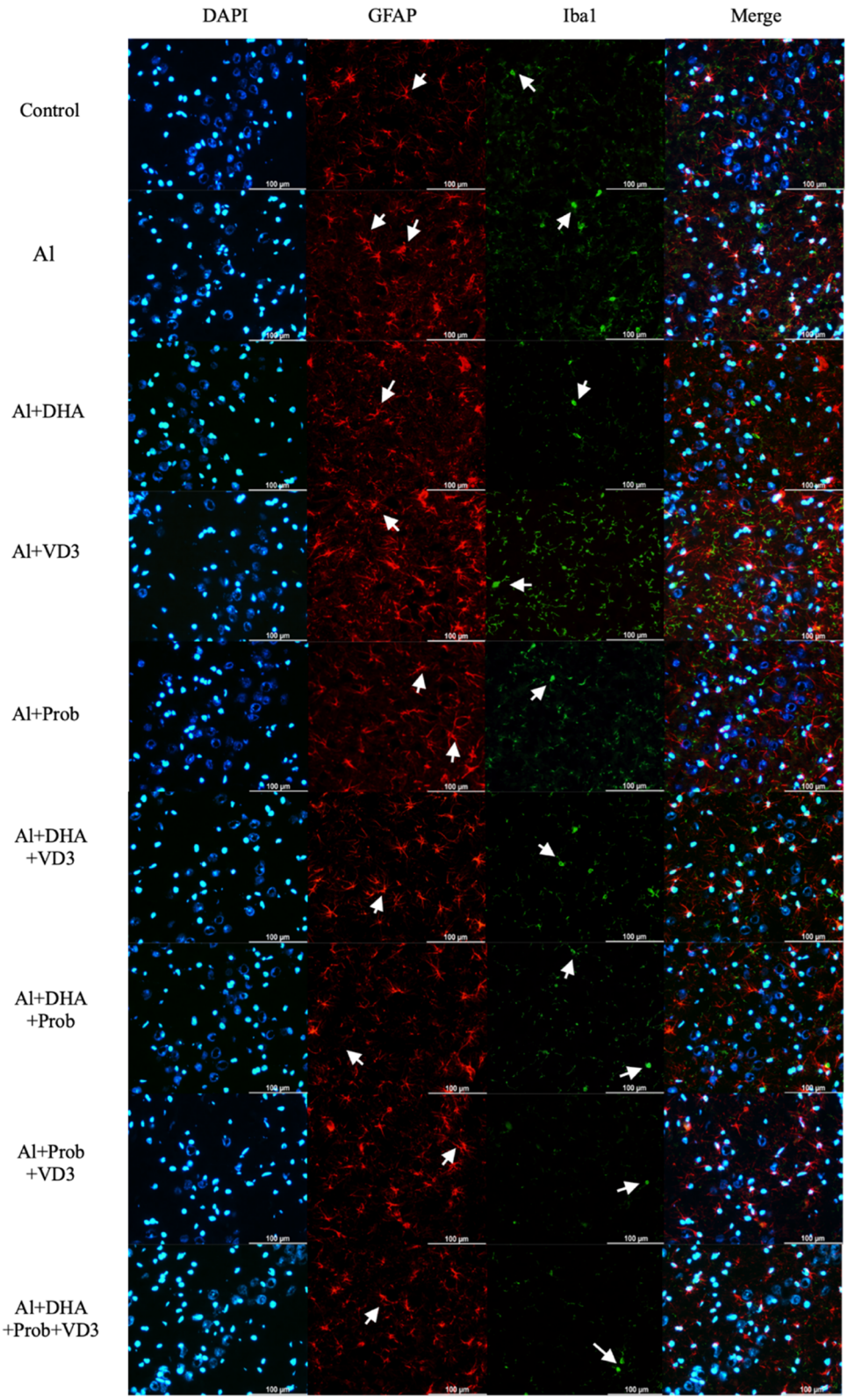

Figure 4. GFAP and Iba1 staining by immunofluorescence in DG hippocampus area. Representative images of GFAP (red) and Iba1 (green) immunostaining in the hippocampus (DG) in Wistar rats under the treatments: Control = healthy individuals; Al = aluminum chloride control (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]), GD = dentate gyrus. DAPI (6-diamidino-2-phenylindole) staining is shown in blue (Magnification 40×, scale bar 100 μM). Arrows denote the activated astrocytes and microglia cells.

represents a socially acceptable intervention. In this line, nutraceuticals have gained popularity due to their potential to boost intelligence, concentration, and memory among cognitive functions.[34] Probiotics (*L. plantarum* [5×10^{10}

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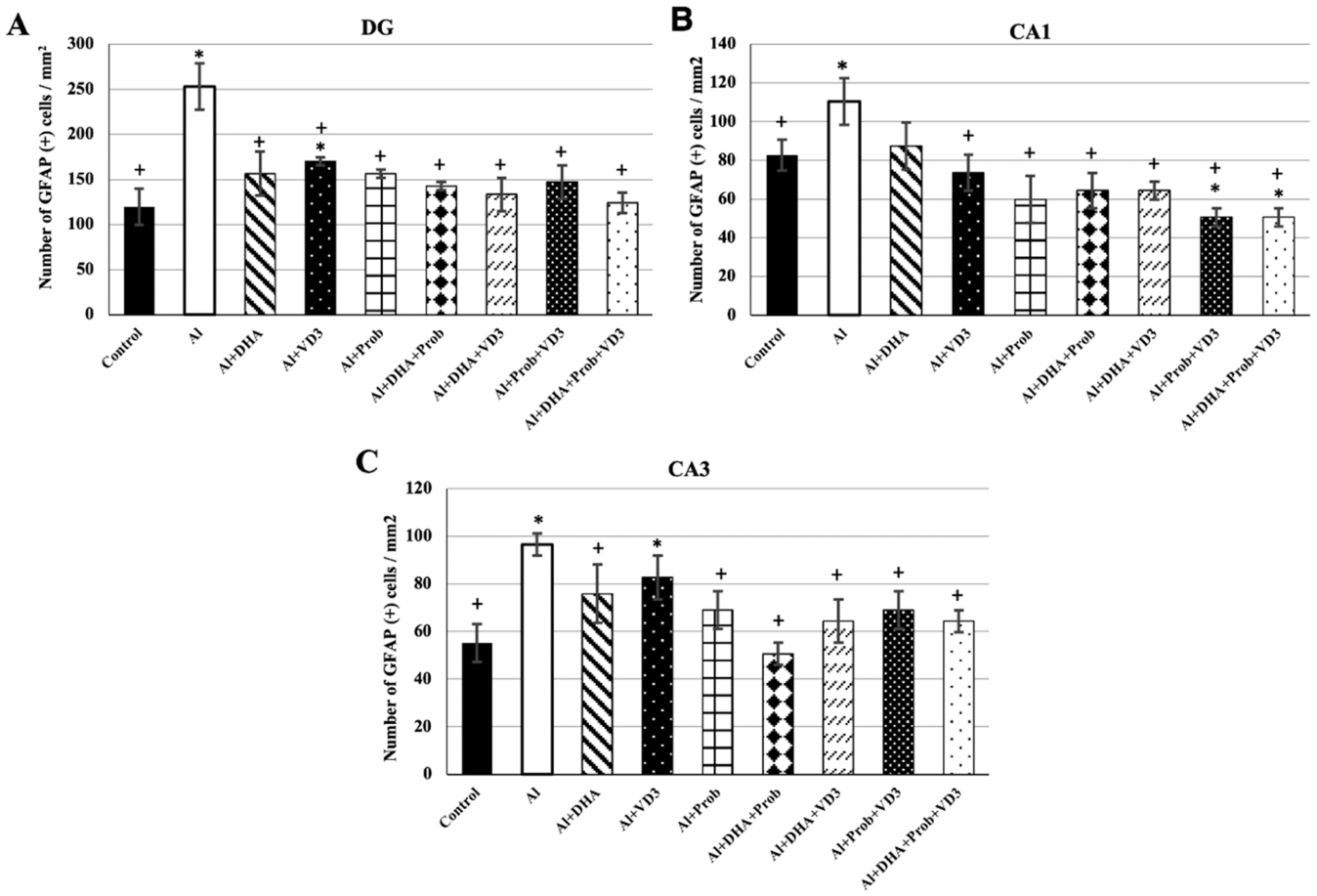

Figure 5. Immunofluorescence analysis of GFAP antibody for the cellular reactivity of astrocytes. Analysis from different hippocampal areas: (A) dentate gyrus (DG), (B) CA1 area, and (C) CA3 area. Cell count was carried out using 40× magnification. Control = healthy individuals; Al = aluminum chloride control (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]). Asterisks (*) indicate a significant difference by the LSD test ($p < 0.05$) compared to the control, and crosses (+) indicate a significant difference by the LSD test ($p < 0.05$) compared to the Al control.

CFU/day], *L. acidophilus* [5×10^{10} CFU/day]), DHA (23.8 mg/day), and VD3 (150 IU/day) were examined in an Al-induced cognitive impairment rat model, given aluminum's role as a neurotoxin associated with neurodegenerative features, including cognitive impairments.[35]

The cognitive impairment model induced by aluminum resulted in the worst performance in the MWM and lowest DI in the NOR test, reflecting spatial, working, and short-term memory problems consistent with Al-induced neurobehavioral changes reported in the literature.[35−42]

Under a preventive scheme, the combined supplementation of DHA + Prob + VD3 showed the most favorable outcomes in the MWM test, exhibiting the lowest swimming distance on the test day, while DHA supplementation led to a higher DI increase in the NOR test. This suggests that nutraceutical administration alleviated cognitive impairment induced by aluminum, supporting their potential in preventing or ameliorating neurodegenerative symptoms.[8]

While research on the combination of nutraceuticals promoting brain plasticity is limited, individual studies have indicated positive effects on cognition, learning, and memory in several neurodegenerative models. DHA acts as a neurotrophic factor, enhancing neuron survival and synaptogenesis.[43] The promotion of these neuroplastic processes impacts cognitive capacity, as demonstrated by Gamoh *et al.* 2001 who reported that in aged rats, the oral administration of DHA improves the performance of the radial arm maze tasks, decreasing the number of reference memory and working memory errors.[44]

VD3 has been associated with protection against cognitive decline, reducing neuronal dystrophy and neuronal loss, and ameliorating working memory deficits. Similarly, the behavioral results from this study agreed with the report by Hou *et al.*,[45] which indicates that treatment with VD3 can improve neuronal survival, enhancing learning, memory, and motor function in an AD transgenic mouse model. Other studies showed that the supplementation of VD3 helped to improve memory performance in the MWM test.[46] This can be associated with the promising role of VD3 in reducing neuronal apoptosis and restoring hippocampal neurogenesis.[47]

Probiotic consumption can influence microbiota composition and impact signaling molecules (neurotransmitters) that regulate the central nervous system and directly influence cognition.[48] The study aligns with findings demonstrating the beneficial effects of probiotics on memory and cognitive function. Jung et al. 2012 reported that oral administration of 1×10^{10} CFU of *Lactobacillus pentosus* var. *plantarum* C29 increased the expression of neurotrophic factors such as p-CREB and BDNF in a scopolamine-induced memory deficit mouse model.[49] The observed improvements in Y-maze and

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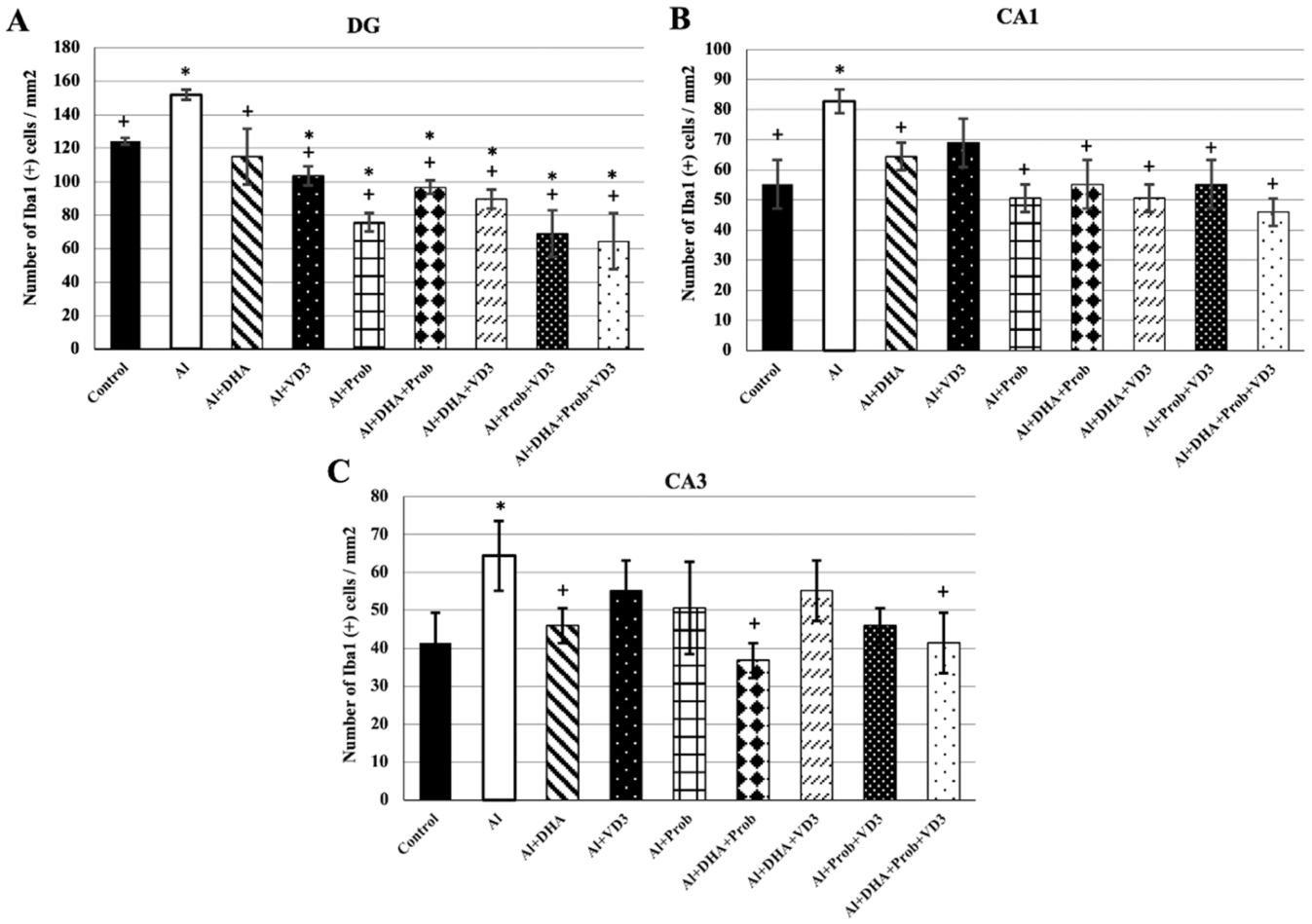

Figure 6. Immunofluorescence analysis of Iba1 antibody for cellular reactivity of microglia. Analysis from different hippocampal areas: (A) Dentate gyrus (DG), (B) Area CA1, and (C) area CA3. Cell count was carried out using 40× magnification in a 233 μm × 311 μm quadrant. Control = healthy individuals; Al = aluminum chloride control (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]). Asterisks (*) indicate a significant difference by the LSD test ($p < 0.05$) compared to the control, and crosses (+) indicate a significant difference by the LSD test ($p < 0.05$) compared to the Al control.

MWM performance align with the increase of these factors. Similarly, Woo *et al.* 2014 reported that *L. pentosus* var. *plantarum* treatment ameliorated the mice D-galactose-induced memory impairment and reversed the suppression of BDNF and DCX expression and CREB activation.[50] The increase in these factors is associated with the improvement of Y-maze and MWM performance.

4.2. Brain Immunofluorescences. Neuroinflammation is a pivotal process in neurodegenerative diseases such as AD and other age-related diseases, and is recognized as a potential mediator of cognitive impairments.[51] Reactive astrogliosis, characterized by increased expression of proteins like glial fibrillary acid protein (GFAP) and vimentin; morphological changes in astrocytes, such as cell soma and processing hypertrophy, along with excessive microglial activation, are implicated in cognitive deficits associated with various diseases.[52,53]

In the cognitive impairment model induced by aluminum (Al), this study observed heightened astrocyte activation (Figure 4), with a more significant process thickening and astrocyte density than the control without Al. This enhanced expression of GFAP-positive cells in the hippocampus indicates reactive astrogliosis.[54,55] Microglial activation, demonstrated by Iba1 immunoreactive cells, further underscored the neuroinflammatory state in critical hippocampal subfields. It is conceivable that Al exposition induced a microglial activation characterized by microglia cells exhibiting hypertrophied and modified processes appearing in clusters with a round-like morphology. The elevated DG, CA1, and CA3 subfield expression of GFAP and Iba-1 observed in this study demonstrated a neuroinflammatory state in this Al-induced cognitive impairment model, concordant perfectly with those previously reported in other studies.[55,56]

Administration of nutraceuticals led to a significant decrease in cell reactivity compared to Al treatment. All treatments reduced the number of GFAP$^+$ cells (Figure 5) in the DG, CA1, and CA3 subfields to a level similar to the control group without Al. The combination of nutraceuticals, particularly DHA + Prob + VD3, demonstrated the most effective reduction in GFAP$^+$ cells. Similarly, nutraceutical combinations showed the best results in reducing Iba1$^+$ cell density in the DG, CA1, and CA3 subfields. DHA + Prob + VD3 exhibited the most significant reduction, suggesting its potential to mitigate microglial activation. Several studies have demonstrated that DHA limits the inflammatory effects likely due to its direct impact on the microglia.[57] The DHA treatment can

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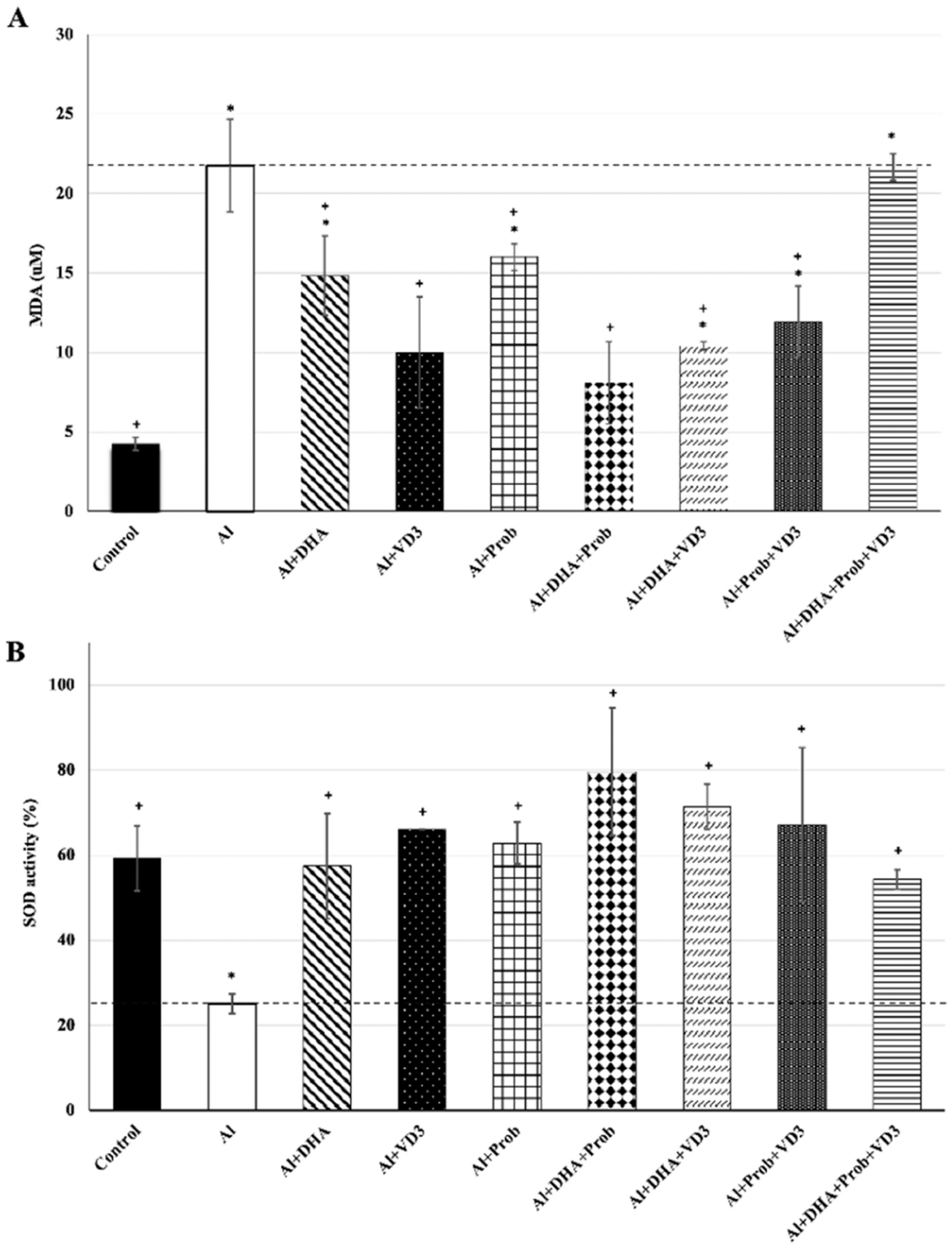

Figure 7. Oxidative stress markers of brain samples. (A) Malondialdehyde (MDA) activity, (B) Superoxide dismutase (SOD) activity. Control = healthy individuals; Al = aluminum chloride control (100 mg/kg.day); DHA = docosahexaenoic acid, life's DHA S17−P100 (23.8 mg/day); VD3 = vitamin D3, dry Vitamin D3 100 SD/S (150 IU/day); Prob = probiotics (*L. plantarum* [5×10^{10} CFU/day], *L. acidophilus* [5×10^{10} CFU/day]). Asterisks (*) indicate a significant difference by the LSD test ($p < 0.05$) compared to the control, and crosses (+) indicate a significant difference by the LSD test ($p < 0.05$) compared to the Al control.

restore the ramification of Iba1 + cells, influencing a morphological shift away from the amoeboid-like shape and toward the classic ramified morphology with cellular extensions protruding from the cell body with a more permissive, surveillant state.[58] Although robust studies regarding probiotics to mitigate neuroinflammation remain under research, it has been proposed that these microorganisms can be used as immunization agents to promote enhanced immunoregulation via T-reg cells, shifting the immune response toward an anti-inflammatory state.[59] Similarly, the VD3 can induce an anti-inflammatory state by shifting the M1 to M2 microglia responses and inhibiting astrocyte activation.[60,61]

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The study's results imply that the nutraceuticals, especially the combination of Al + DHA + Prob + VD3, played a role in attenuating reactive astrogliosis and microglial activation, potentially contributing to neurodegeneration repair and functional behavioral improvements.

4.3. Oxidative Stress Markers. The brain, a highly enriched organ in polyunsaturated fatty acids, is sensitive to free radicals following toxic insults, such as aluminum (Al) exposure.[52] This often leads to increased lipid peroxidation, notably measured by malondialdehyde (MDA), a crucial marker reflecting cellular oxidative stress. Additionally, antioxidant enzyme activities, including glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT), decrease in response to Al exposure, further indicating oxidative stress state.[54,62]

Consistent with previous studies, Al exposure in this study elevated MDA levels and reduced SOD activity, confirming induced oxidative stress in rat brains.[54,62,63] However, the administration of nutraceuticals resulted in a significant decrease in MDA levels, with the DHA + Prob combination exhibiting a more pronounced effect than individual DHA and Prob supplementation, supporting the notion that combining these nutraceuticals enhances its bioactivities.[64] Intriguingly, VD3 alone also displayed notable efficacy in reducing the MDA concentration. Similar results are showed in others studies where supplementation of VD3 showed a significant decrease of MDA values in a LPS-induced cognitive impairment and anxiety/depression model.[65,66]

In terms of SOD activity, the DHA + Prob treatment demonstrated the highest percentage, surpassing even that of the healthy control. However, combining VD3 with DHA + Prob appeared to moderate the elevated SOD activity seen with DHA + Prob, possibly due to interactive effects producing an antagonistic response.[64]

The observed protective effect of DHA aligns with existing literature, suggesting that increased DHA levels in the brain may confer protection against oxidative stress.[67] DHA's indirect antioxidant role involves modulating the expression/activity of various proteins, including Nrf2, GPx, and SOD, and scavenging intracellular reactive oxygen species (ROS) in nervous tissues.[68,69] While more research is needed to elucidate the underlying mechanisms, the study suggests that supplementing nutraceutical combinations can mitigate Al-induced oxidative stress through their antioxidant and free radical-scavenging effects. This underscores their potential therapeutic value in addressing oxidative stress-related complications in the brain.[70,71]

5. CONCLUSIONS

Nutraceuticals are a promising strategy for preventing and treating neurodegenerative disorders. They are nontoxic, nonaddictive, and have bioactivities that promote neurological well-being. This study showed that some nutraceuticals and their combinations positively affect cognitive impairment by acting as immunomodulators and antioxidants and ameliorating aluminum-induced cognitive impairment. These benefits encompassed memory enhancement, diminished MDA concentration, increased SOD activity, and reduced glial activation as indicated by GFAP/Iba1 markers.

While the current findings showcase promising outcomes, exploring a broader spectrum of nutraceutical combinations on the progression of neurodegenerative diseases is essential. Focusing on cognitive impairment and subjacent neuropathological processes and incorporating robust neurobiological markers and behavioral tests is also important, as it can offer valuable insights into potential nutraceutical mechanisms of action. To enhance the research's translational potential, nutraceuticals' efficacy should be explored in more sophisticated models, such as transgenic or accelerated aging models. We can significantly advance our understanding of nutraceutical interventions by adopting a specific focus on cognitive impairment and incorporating these advanced models. This, in turn, can pave the way for their seamless integration into practical healthcare approaches, ultimately benefiting patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c01198.

GFAP and Iba1 staining by immunofluorescence in CA1 hippocampus area (Figure S1); GFAP and Iba1 staining by immunofluorescence in CA3 hippocampus area (Figure S2) (PDF)

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Author Contributions

P.F.-B., D.J.-V., and E.E.R.-Z.: conceptualization, methodology, and writing. P.F.-B. and E.E.R.-Z.: formal analysis. P.F.-B., E.E.R.-Z., and D.A.C.-P.: experiments. D.J.-V. and A.A.C.-A.: resources. P.F.-B., D.J.-V., and E.E.R.-Z.: writing−original draft preparation. D.J.-V. and E.E.R.-Z.: supervision. All authors have read and agreed to the published version of the manuscript.

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Notes

The authors declare no competing financial interest.

Ethics Statement This animal study was reviewed and approved by the Internal Committee for the Care and Use of Laboratory Animals (Code: 2022−015A).

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