Journal of Functional Foods 100 (2023) 105363

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Synergistic effect of n-3 PUFA and probiotic supplementation on bone loss induced by chronic mild stress through the brain–gut–bone axis

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ARTICLE INFO

Keywords:
Brain–gut–bone axis
Chronic mild stress
Depression
N-3 polyunsaturated fatty acid
Osteoporosis
Probiotics

ABSTRACT

This study was to investigate the synergistic effect of n-3 polyunsaturated fatty acids (PUFA) and probiotics on depression-induced bone loss. Rats were fed with a diet composed of 0% or 1% n-3 PUFA and live or dead probiotics, and underwent chronic mild stress. N-3 PUFA with live or dead probiotics enhanced bone mass, calcium, and osteoprotegerin and decreased amino-terminal cross-linked telopeptide of type 1 collagen, receptor activator of nuclear factor kappa-B ligand, and cytokines. N-3 PUFA and probiotics increased brain serotonin and modulated the hypothalamic–pituitary–adrenal axis, thereby decreasing the bone expression of beta 2 adrenergic receptor and activating transcription factor 4 and increasing runt-related factor 2. N-3 PUFA and probiotics decreased gut serotonin, which downregulated the bone expression of 5-hydroxytryptamine receptor 1B and increased cyclic adenosine monophosphate. This study showed the synergistic bone-protective effects of n-3 PUFA and probiotics through the brain–gut–bone axis.

1. Introduction

Osteoporosis is characterized by the decreased bone mass and increased bone fragility, which is associated with depression, suggesting the bone–brain connection (Cizza et al., 2010). Recent understanding of the gut microbiome has shown that restoring the balance of the microbiome is closely related to osteoporosis and has provided a theoretical basis for the brain–gut–bone axis as a driving force of the progression of osteoporosis (Zhang et al., 2022).

Depression induced by chronic mild stress (CMS) decreases bone volume in rats (Choi et al., 2022; Henneicke et al., 2017); patients with depression have a lower spine bone mass than healthy controls (Schweiger et al., 1994). CMS of multiple stressors during 2 weeks downregulates neuronal serotonin responses and enhances the neuroendocrine hypothalamic–pituitary–adrenal (HPA) axis system, including adrenocorticotropic hormone (ACTH) and corticosterone (CORT) (Choi et al., 2022). The upregulated HPA axis enhances beta 2 adrenergic receptor (ADRβ2), which decreases bone formation and increases bone resorption (Collet et al., 2008; Ducy & Karsenty, 2010). Conversely, CMS of multiple stressors during 4 weeks increases the level of gut-derived serotonin, which binds to serotonin 1B receptor (5-HT_{1b}R) (Li et al., 2019), inhibits cyclic adenosine monophosphate (cAMP) signaling, and decreases bone formation (Yadav et al., 2008).

Our previous studies show that n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), but not α-linolenic acid (ALA), improve depressive behaviors measured including anhedonia and immobility by sucrose preference test (SPT) and forced swimming test (FST), and bone mass in stressed male rats (Brenes Sáenz et al., 2006; Choi et al., 2021). EPA and DHA upregulates the brain derived-serotonin pathway and downregulates the HPA axis in depressed rats (Choi et al., 2021; Choi & Park, 2017; Kim et al., 2020). Serotonergic pathways and HPA axis ameliorate bone loss through the modificating bone transcription factors such as activating transcription factor 4 (ATF4) and runt-related factor 2 (RUNX2), which lead to an increase in the ratio of osteoprotegerin (OPG)/receptor activator of nuclear factor kappa-B ligand (RANKL), and decrease in bone-resorbing

Abbreviations: ACTH, adrenocorticotropic hormone; ADRβ2, adrenergic β2 receptor; ATF4, activating transcription factor 4; BMC, bone mineral content; BMD, bone mineral density; cAMP, cyclic adenosine monophosphate; CMS, chronic mild stress; CORT, corticosterone; DAPI, 4′, 6-diamidino-2- phenylindole; DHA, docosahexaenoic acid; DXA, dual energy X-ray absorptiometry; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FST, forced swimming test; HPA, hypothalamic–pituitary–adrenal; IL-1β, interleukin-1β; NTx, amino-terminal cross-linked telopeptide of type 1 collagen; OPG, osteoprotegerin; OVX, ovariectomized; PUFA, polyunsaturated fatty acids; RANKL, receptor activator of nuclear factor kappa-B ligand; RUNX2, runt-related transcription factor 2; SPT, sucrose preference test; TBST, Tris-buffered saline with 0.1% Tween 20; TNF-α, tumor necrosis factor-α; 5-HT_{1b}R, serotonin 1B receptor.

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https://doi.org/10.1016/j.jff.2022.105363
Received 21 September 2022; Received in revised form 22 November 2022; Accepted 3 December 2022
Available online 6 December 2022

cytokines in ovariectomized (OVX) and depressed rats (Choi et al., 2022; Jin et al., 2017; Oury et al., 2010). Fish oil also reduces the gut levels of inflammatory cytokines, which modulate the bone mass of the femur in OVX rats, suggesting the effect of n-3 PUFA on the gut–bone axis (Zhu et al., 2021). However, studies have yet to determine whether the effect of n-3 PUFA on the bone is related to the gut-derived serotonin pathway.

Mixture of *Bifidobacterium longum, Lactobacillus helveticus*, and *Lactobacillus Plantarum* has bone-protecting and antidepressant effects by improving SPT and FST (Collins et al., 2017; Li et al., 2018). The supplementation of live probiotics containing *Lactobacillus helveticus, Lactobacillus plantarum, and Bifidobacterium longum* reduce gut-derived serotonin levels, increase brain-derived serotonin levels in CMS rats (Li et al., 2018), and elicits preventive and therapeutic effects on bone mass in chronically stressed rats (Yadav et al., 2008). Live *Lactobacillus reuteri* modifies gut microbiota, which downregulates inflammatory cytokines and RANKL but upregulates OPG in OVX mice (Britton et al., 2014; Ohlsson et al., 2014). Live and dead *Lactobacillus reuteri* reduce the alveolar bone loss of rats with periodontitis (Moraes et al., 2020; Yu & Wang, 2022), and live and dead *Lactobacillus casei* downregulate inflammatory cytokines in bone dendritic cells (Thakur et al., 2016). Conversely, Liu et al. (2016) reported that live *Lactobacillus plantarum* but not dead improves anxiety-like behaviors and increase brain-derived serotonin and dopamine levels in germ-free mice; therefore, live and dead probiotics may have different effects on depressed conditions.

EPA and DHA, and probiotics synergistically ameliorate the histomorphometric appearance of alveolar bone loss and decrease the serum levels of inflammatory cytokines compared with that of their single use in the ligature-induced periodontitis of rats (Doğan et al., 2022). However, studies have yet to examine the synergistic mechanisms of n-3 PUFA and probiotics on bone loss. Therefore, this study was performed to investigate the synergistic effect of n-3 PUFA and probiotics on CMS-induced bone loss through the brain–gut–bone axis, and examine whether dead probiotics had similar bone protection effects as live probiotics.

2. Materials and methods

2.1. Animals and diet

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-21-0092). Four-week-old male Wistar rats (Central Lab, Animal Inc., Seoul, Korea) were housed in a group of three and kept in a ventilated air-conditioned room maintained at 22 ± 1 °C with a 12 h/12 h light/dark cycle and 40 %–50 % humidity. Experimental diets were the isocaloric-modified American Institute of Nutrition-93G diets containing 16 % fat of the total energy with 0 % n-3 PUFA or n-3 PUFA of the total energy (Table S1). The n-3 PUFA deficient diet was made of grape seed oil (Sajo Haepyo, Seoul, Korea), and the n-3 PUFA diet was made of a combination of grape seed oil and re-esterified triglyceride fish oil (Epax, Ålesund, Norway) containing 0.6 % EPA and 0.4 % DHA from the total energy of diet. Live and dead probiotics were prepared in a mixture consisting of *Bifidobacterium longum*, *Lactobacillus helveticus*, and *Lactobacillus plantarum* (1:1:1; Lactomason, Jinju, Korea). The rats were administered orally with 1 mL of sterile water with or without 6×10^9 colony-forming units of probiotics daily.

2.2. Experimental design

After 1 week of acclimatization, the rats consumed the experimental diet and supplementation of probiotics for 12 weeks until the end of the study (Fig. S1A). The rats were randomly divided into seven groups (n = 8): NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS.

CMS was started on 11-week-old rats for 5 weeks (Fig. S1B). The CMS procedure involved food deprivation, water deprivation, cage tilting (45°), soiled cage (300 mL of water with 25 ± 1 °C), group housing (6 rats per cage), no bedding, stroboscopic illumination (130 flashes/min), and intermittent illumination every 2 h (Bravo, 2012), to avoid adaptation (de Mello et al., 2014). The rats of the NS group were housed separately from those of the CMS group under similar conditions, but they were not disturbed during CMS. SPT and FST were performed at the end of the study.

2.3. Behavioral tests

SPT was conducted to measure the core symptom of depression anhedonia (Eagle et al., 2016). Before SPT, pre-SPT was performed for 4 days; during pre-SPT, the rats were exposed to two bottles containing either sterile water or 1 % sucrose solution for adaptation. The placement of the bottles was switched each day to avoid side bias, and the bottles were weighed daily. In SPT, two bottles containing either sterile water or 1 % sucrose solution were administered after 10 h of food and water deprivation. The sucrose preference index (%) was calculated as (grams of sucrose solution consumption/total grams of liquid consumption) × 100.

The day before being euthanized, the rats were subjected to FST to measure their depression-related behavior (Kim et al., 2020). They were individually placed inside a cylinder (height 50 cm, diameter 20 cm) containing water (25 ± 1 °C) up to a height of 30 cm. During pre-FST, the rats were forced to swim for 15 min; after 24 h, each rat was re-exposed to FST for 5 min. FSTs were performed using a video camera positioned in front of the cylinders. The durations of immobility, swimming, and climbing were measured by three raters who were unaware of the test conditions.

2.4. Blood and tissue collection

The rats were anesthetized using Zoletil (30 mg/kg) and Rompun (10 mg/kg). Serum and plasma were collected, and dissected organs were rinsed with saline, weighed, and frozen in liquid nitrogen immediately. Blood and tissue samples were stored at − 80 °C for further analysis.

2.5. Levels of hormones and bone markers

The concentrations of CORT (Enzo Life Science, NY, USA) and ACTH (Phoenix Pharmaceuticals, CA, USA) in plasma, amino-terminal cross-linked telopeptide of type 1 collagen (NTx; Alere, MA, USA) in serum, and serotonin in the brain and gut (Aviva Systems Biology Corp., San Diego, CA, USA) were analyzed using enzyme-linked immunosorbent assay kits. The calcium concentration of the bone was analyzed using colorimetric assay kits (Abcam, Cambridge, UK). All measurements were performed in duplicate in accordance with the manufacturer's instructions and quantified against authentic standards by using a spectrophotometer (Multiscan GO, Thermo Scientific, MA, USA). Under anesthesia, the bone mineral density (BMD; g/cm^2) and bone mineral content (BMC; g) in the right femur and tibia were measured through dual-energy X-ray absorptiometry (DXA; InAlyzer, Medikors Co., Seongnam, Korea).

2.6. Western blot analysis

Femoral tissues were homogenized in radioimmunoprecipitation buffer (Sigma-Aldrich, St. Louis, MO, USA) containing complete EDTA-free protease and PhosSTOP inhibitor (Roche Diagnostics GMbH, Mannheim, Germany). Then, they were centrifuged at 10,000*g* and 4 °C for 15 min. A bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL,

USA) was used to quantify proteins in the supernatants of femoral tissues. Proteins (30 μg) in the supernatants of each tissue were separated through 10 %–12 % SDS-PAGE, transferred to polyvinylidene fluoride membranes, and blocked with 5 % skim milk or bovine serum albumin in Tris-buffered saline with 0.1 % Tween 20 (TBST) at room temperature for 1–2 h. The membrane was then incubated with the primary antibodies against RUNX2 (1:1000), RANKL (1:2000), OPG (1:1000), ADRβ2 (1:2000), ATF4 (1:1000), cAMP (1:1000), IL-1β (1:500), tumor necrosis factor-α (TNF-α; 1:500), and 5-HT_{1b}R (1:500) with 5 % skim milk or 3 %–5% bovine serum albumin in TBST at 4 °C overnight. Antibodies against ADRβ2 and ATF4 were purchased from Bioss (Woburn, MA, USA) and Invitrogen (Carlsbad, CA, USA), respectively, and the other antibodies were purchased from Abcam (Cambridge, UK). After being washed with TBST, the membranes were incubated with a horseradish-peroxidase-conjugated secondary antibody (either anti-rabbit IgG [Cell signaling, New England Biolabs, Beverly, MA, USA] or anti-mouse IgG [Enzo Life Science, Farmingdale, NY, USA]) with 5 % skim milk or 3 %–5% bovine serum albumin in TBST at room temperature for 1 h. Immunoreactive bands were visualized with a UV setting on a Chemidoc MP Imaging System (Bio-rad, Hercules, CA, USA) to estimate the total protein per lane, and tubulin was used for normalization. Western blot analysis was performed in duplicate.

2.7. Immunofluorescence staining

The rats were anesthetized and perfused with saline and 4 % paraformaldehyde through the aorta. The right femurs were dissected, decalcified with 10 % EDTA (pH 7.4), and fixed with 4 % paraformaldehyde. The bones were dehydrated, embedded in paraffin, cut into 4 μm sections, and mounted on glass slides. The sections were immersed initially in xylene and then in alcohol and rehydrated in wash buffer. Antigens were retrieved by heating the sections in sodium citrate buffer (0.01 M, pH 6.0). After the sections were blocked with PAP pen (Enzo Life Science, Farmingdale, NY, USA), anti-5-HT_{1b}R (1:100; Sigma-Aldrich, St. Louis, MO, USA) and anti-ADRβ2 (1:100; Bioss, Woburn, MA, USA) were added and incubated at 4 °C overnight. The sections were incubated with goat anti-rabbit IgG H&L with DyLight® 488 (Abcam, Cambridge, UK) at room temperature for 2 h, washed, and mounted with Fluoroshield containing 4′,6-diamidino-2-phenylindole (DAPI; Abcam, Cambridge, UK). A Leica TCS SP8 X confocal microscope and Leica AF imaging (Leica Microsystems, Wetzlar, Germany) were used to evaluate immunofluorescence images.

2.8. Gas chromatography

The left femur (100 mg) was homogenized, total fats were extracted, and phospholipids were separated using thin-layer chromatography (Machereynagel GmbH & Co, Duren, Germany). Phospholipids were methylated, and the composition of long chain fatty acids (14:0 or more) was analyzed using gas chromatography (Shimadzu Scientific Instrument, Tokyo, Japan). Hydrogen gas was used as the carrier gas, and the injection and detection temperatures were 230 and 240 °C, respectively. The run temperature began at 180 °C, and the split ratio was 10:1 and external standards (Nu-Check Prep Elysian, MN, USA) were used for identification of fatty acids; the coefficient of variation was 4.3 %.

To analyze short chain fatty acid (SCFA) levels, an internal standard (7:0; Sigma-Aldrich, Burlington, MA, USA) was added during homogenization of the gut tissue, and then, NaOH and phosphoric acid were added for stabilization and acidification. The SCFAs levels, including acetic (2:0), propionic (3:0), isobutyric (4:0 *i*), and butyric acids (4:0*n*), were analyzed (Supelco, Bellefonte, PA, USA). Carrier gas was nitrogen, and the injection and detection temperatures were 190 °C. The run temperature began at 80 °C, and the split ratio was 10:1. To identify the fatty acids, the external standards (Matreya, State College, PA, USA) were used.

2.9. Statistical analysis

Data were analyzed using SPSS for Windows version 27.0 (SPSS Inc., Chicago, IL, USA). Values were expressed as mean and standard deviation, and differences were considered significant at $P < 0.05$. They were analyzed using independent *t*-test with stress as a factor. Two-way analysis of variance (ANOVA) was performed to analyze the individual and synergistic effects of diet and probiotics. Then, Duncan's *post hoc* test was conducted to determine the differences between live and dead probiotics.

3. Results

3.1. Depressive behaviors

CMS significantly decreased the final body weight, but CMS, diet, and probiotics had no significant effects on dietary intake and organ weight (Table 1). CMS significantly decreased the sucrose preference index, swimming, and climbing time but increased immobility time ($P < 0.001$; Fig. 1). N-3 PUFA diet significantly increased the sucrose preference index and climbing time but decreased immobility time ($P < 0.001$). Probiotics significantly increased the sucrose preference index and climbing time ($P < 0.001$), but no differences were observed between the effects of live and dead probiotics on depressive behaviors. N-3 PUFA diet and probiotics synergistically affected climbing time ($P = 0.039$).

3.2. Bone markers and levels of hormones

CMS significantly increased the blood level of NTx and decreased the bone level of calcium, BMC, and BMD of the femur and tibia ($P < 0.001$; Fig. 2). N-3 PUFA diet and probiotics significantly decreased the blood level of NTx and increased the bone level of calcium, BMC, and BMD of the femur and tibia. However, these levels had no differences between live and dead probiotics. N-3 PUFA diet and probiotics elicited a significant synergistic effect on bone markers and bone mass.

Consistent with changes in behaviors, CMS significantly increased the levels of ACTH and CORT in the blood and the level of serotonin in the gut; conversely, it decreased the brain level of serotonin ($P < 0.001$; Fig. 3). N-3 PUFA diet and probiotics significantly decreased the levels of ACTH and CORT and the levels of serotonin in the gut but increased the brain level of serotonin. However, these levels had no differences between live and dead probiotics. N-3 PUFA diet and probiotics had a significant synergistic effect on hormone levels.

3.3. Protein expression of bone-related markers

CMS significantly increased the expression of ADRβ2, ATF-4, RANKL, and 5-HT_{1b}R but decreased the expression of OPG, and RUNX2 ($P < 0.001$; Fig. 4). CMS significantly increased the levels of TNF-α, and IL-1β but decreased the levels of cAMP. N-3 PUFA diet and probiotics significantly decreased the expression of ADRβ2, ATF-4, RANKL, and 5-HT_{1b}R, but they increased the expression of OPG, and RUNX2 ($P < 0.001$). N-3 PUFA diet and probiotics significantly decreased the levels of TNF-α, and IL-1β, but they increased the levels of cAMP ($P < 0.001$). The supplementation of n-3 PUFA diet and probiotics synergistically affected the protein expression and levels of bone-related markers ($P < 0.001$). However, no significant differences were observed between live and dead probiotics. Consistently, immunofluorescence staining showed that CMS increased, but the supplementation of n-3 PUFA diet and probiotics decreased the expression of ADRβ2 and 5-HT_{1b}R in the femoral tissue (Fig. 5).

3.4. Fatty acids of bone and gut

CMS and probiotics had no effect on the bone phospholipid

Table 1
Dietary intake, body weight, and organ weight.[1] *Values are significantly different between NS and N3D.

	NS	CMS						*P*-value[2]			
		N3D	LP	DP	N3	LPN3	DPN3	CMS	Diet	Probiotics	Diet× Probiotics
Dietary intake (g/day)	19.75 ± 1.57	20.60 ± 0.57	19.46 ± 0.88	20.27 ± 1.17	20.46 ± 0.85	19.97 ± 0.54	21.00 ± 0.70	0.105	0.359	0.141	0.638
Initial body weight (g)	158.79 ± 19.44	153.43 ± 12.65	150.37 ± 6.67	149.70 ± 11.06	147.04 ± 6.23	149.55 ± 12.55	149.51 ± 18.68	0.500	0.437	0.987	0.685
Final body weight (g)	555.76 ± 19.31	435.86 ± 33.48	455.14 ± 20.76	461.16 ± 36.96	456.91 ± 39.34	472.79 ± 19.57	459.49 ± 52.62	0.022	0.237	0.347	0.626
Brain (g)	0.29 ± 0.03	0.29 ± 0.02	0.30 ± 0.02	0.29 ± 0.02	0.29 ± 0.03	0.29 ± 0.03	0.28 ± 0.01	0.250	0.278	0.302	0.912
Gut (g)	0.44 ± 0.15	0.38 ± 0.05	0.34 ± 0.07	0.39 ± 0.06	0.37 ± 0.09	0.40 ± 0.11	0.42 ± 0.06	0.112	0.400	0.180	0.443
Femur (g)	2.38 ± 0.36	2.29 ± 0.31	2.35 ± 0.41	2.15 ± 0.38	2.26 ± 0.37	2.16 ± 0.22	2.14 ± 0.31	0.155	0.487	0.311	0.928
Tibia (g)	1.68 ± 0.15	1.65 ± 0.22	1.71 ± 0.17	1.62 ± 0.17	1.70 ± 0.16	1.71 ± 0.18	1.76 ± 0.13	0.298	0.538	0.457	0.530

[1] Values are expressed as mean and standard deviation; NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS.

[2] Independent *t*-test was used to determine CMS effect; diet effect was determined by two-way ANOVA, and probiotic effect was determined by two-way ANOVA followed by Duncan's *post hoc* test.

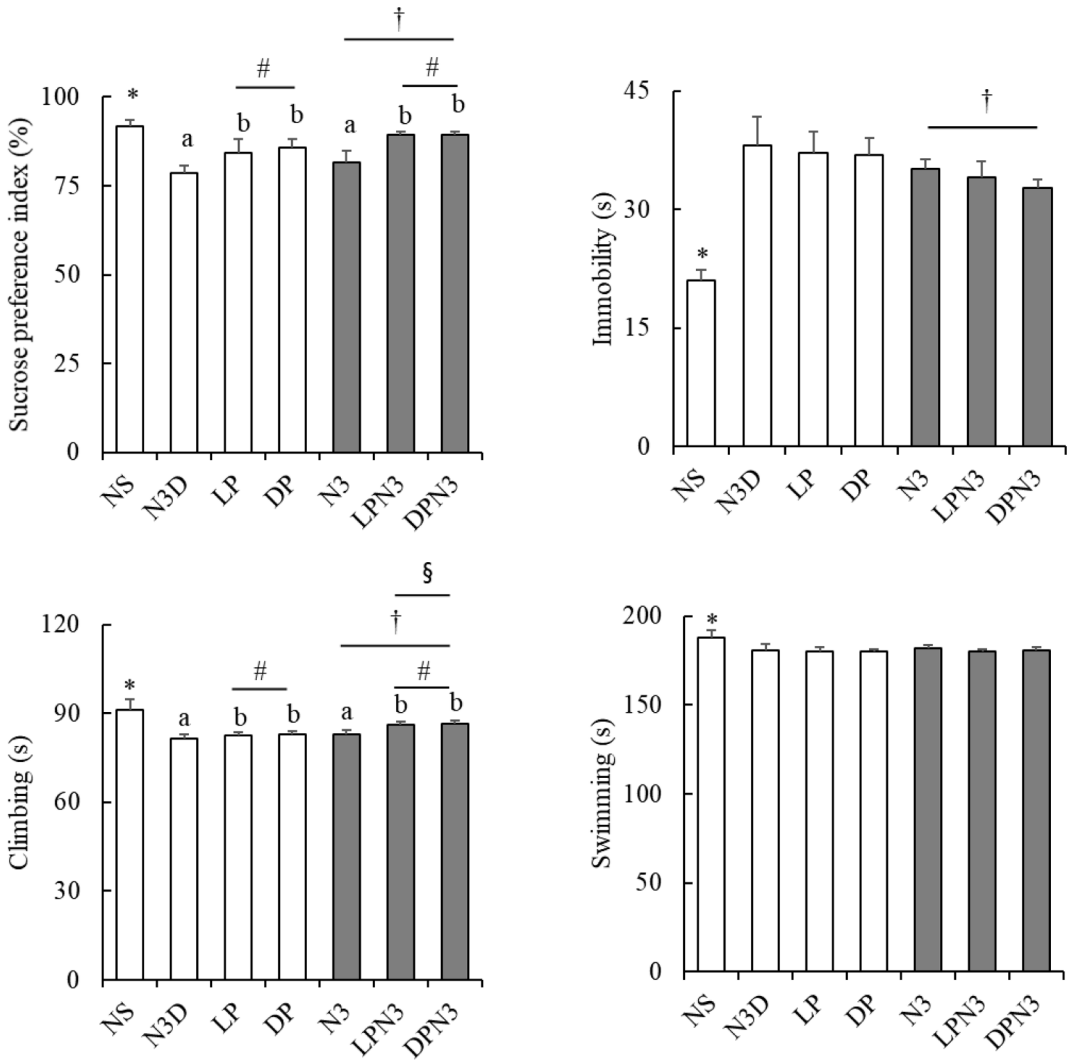

Fig. 1. Depressive behaviors measured with a sucrose preference test and a forced swimming test NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; DPN3, n-3 PUFA diet and dead probiotics with CMS (n = 8). *Values are significantly different between NS and N3D; #Values are significantly different between probiotics and nonprobiotics; [a-b]Values with different letters are significantly different between live and dead probiotics within the same diet; †Values are significantly different between n-3 PUFA and n-3 PUFA deficient diets; §Values show the significant synergistic effect of n-3 PUFA diet and probiotics.

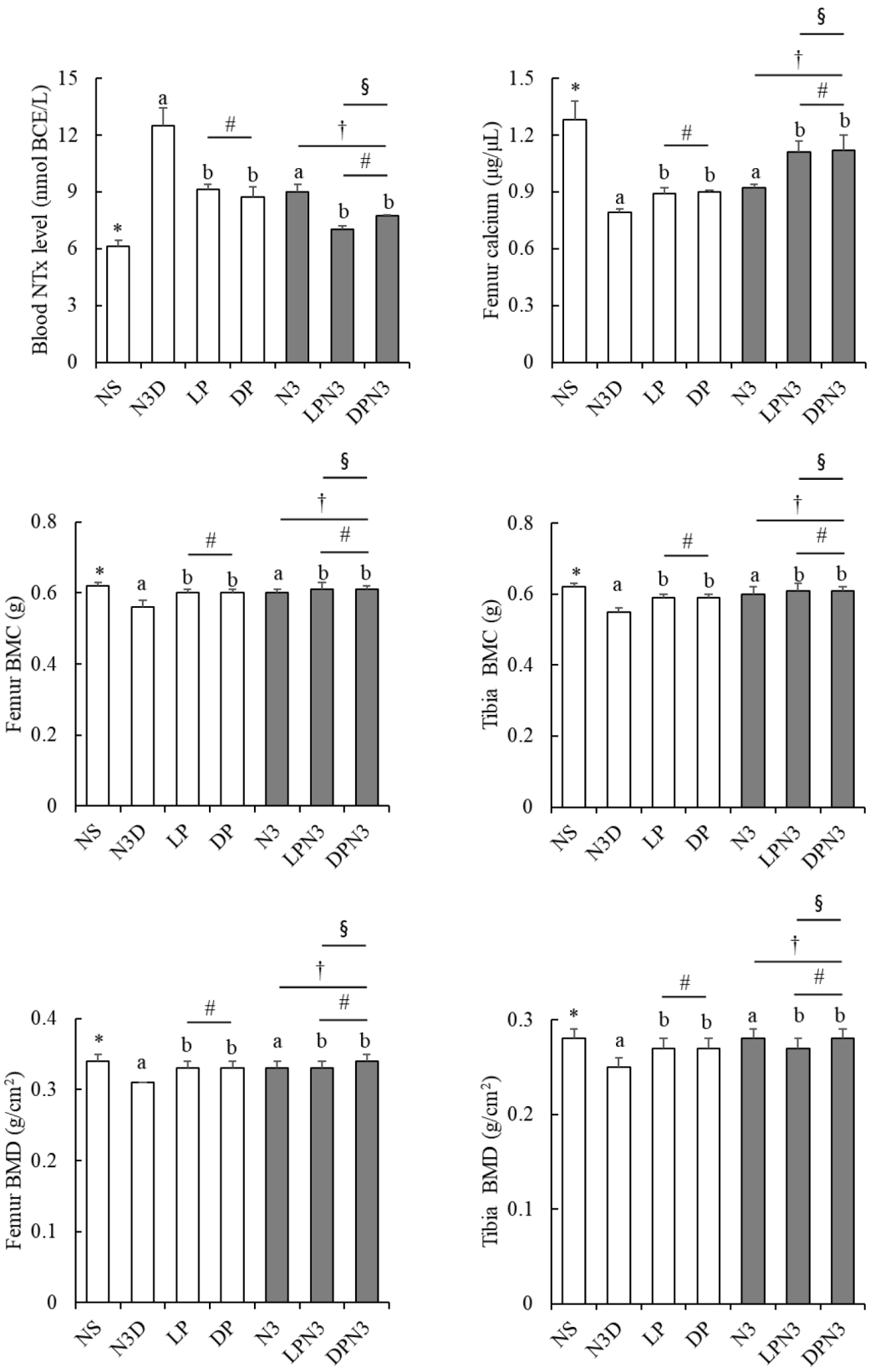

Fig. 2. Bone turnover markers and bone mass NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS (n = 8); NTx, amino-terminal cross-linked telopeptide of type 1 collagen; Ca, calcium; BMC, bone mineral content; BMD, bone mineral density. *Values are significantly different between NS and N3D. #Values are significantly different between probiotics and nonprobiotics; [a-b]Values with different letters are significantly different between live and dead probiotics within the same diet; †Values are significantly different between n-3 PUFA and n-3 PUFA deficient diets; §Values show the significant synergistic effect of n-3 PUFA diet and probiotics.

composition of long chain fatty acids (Table 2). Supplementation of n-3 PUFA significantly increased 20:5n3, 22:5n3, 22:6n3, and total n-3 PUFA but decreased 18:2n6, 20:4n6, 22:4n6, 22:5n6, and total n-6 PUFA. There was a synergistic effect of the n-3 PUFA diet and probiotics supplementations on 20:5n3 in the bone.

CMS significantly decreased the levels of acetic, propionic, isobutyric, and butyric acids in the gut (Table 3). Supplementation of n-3 PUFA and probiotics significantly increased the gut levels of acetic, propionic, and butyric acids but not isobutyric acid. There were significant synergistic effects on the gut levels of acetic, propionic, and butyric

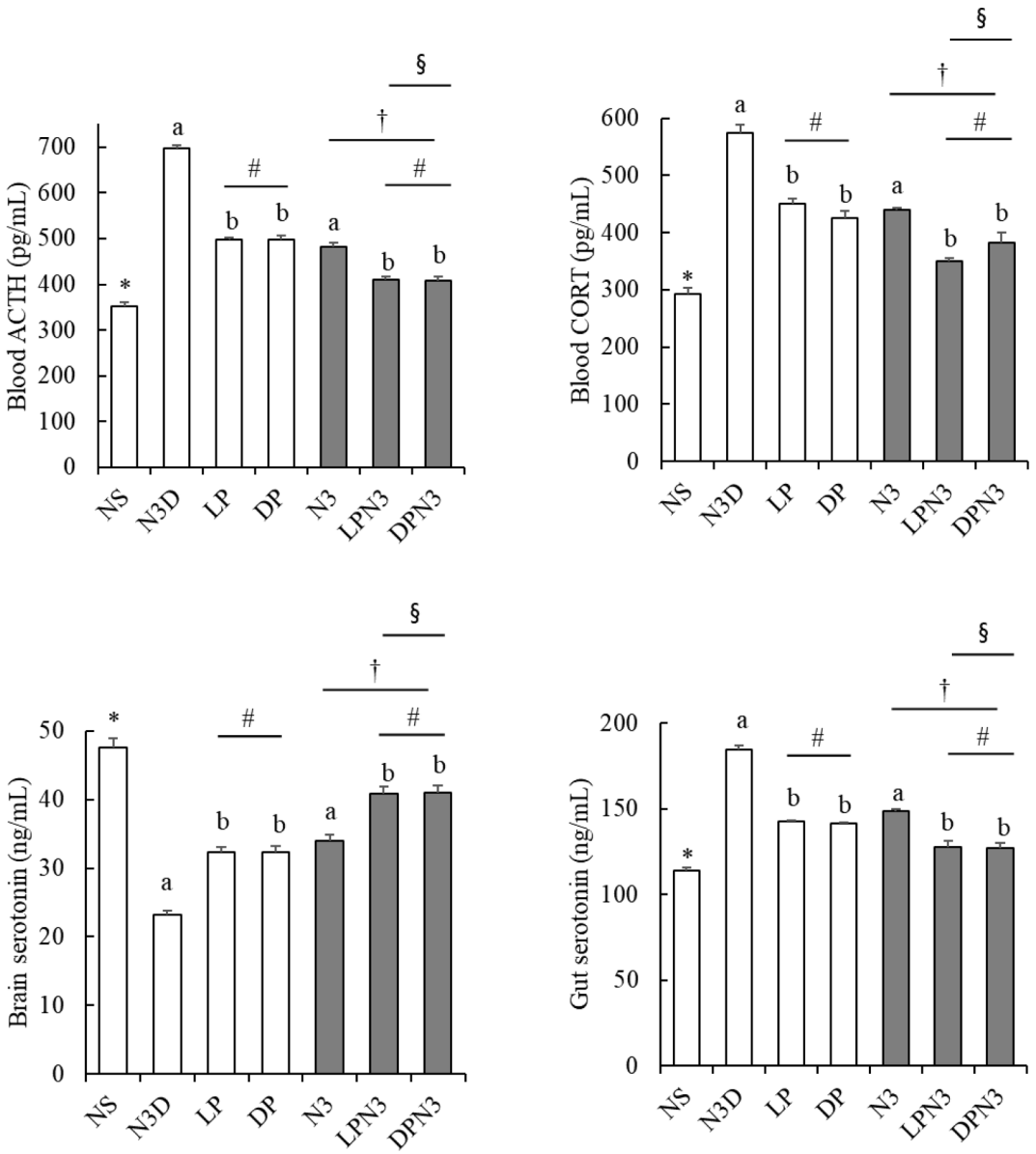

Fig. 3. Levels of hormones in the blood, brain, and gut NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS (n = 8); ACTH, adrenocorticotropic hormone; CORT, corticosterone. *Values are significantly different between NS and N3D. #Values are significantly different between probiotics and nonprobiotics; a-bValues with different letters are significantly different between live and dead probiotics within the same diet; †Values are significantly different between n-3 PUFA and n-3 PUFA deficient diets; §Values show the significant synergistic effect of n-3 PUFA diet and probiotics.

acids, but no significant differences between live and dead probiotics on the gut levels of SCFA.

4. Discussion

This study demonstrated that the supplementation of n-3 PUFA and probiotics synergistically ameliorated the CMS-induced bone loss through the brain–gut–bone axis. The supplementation of n-3 PUFA and probiotics upregulated the brain-derived serotonin pathway but downregulated the HPA axis activity and gut-derived serotonin pathway. Previous studies reported that depression decreases bone mass in rats exposed to stress such as CMS and early life maternal separation (Choi et al., 2022). CMS, an animal model for depression, increases the blood level of NTx (a bone resorption marker), and it decreases the bone levels of calcium (a bone formation marker) (Choi et al., 2022). CMS also downregulates brain-derived serotonin and hyperactivates the HPA axis system, which increases bone resorption by modulating the RANKL-related bone transcription factors of osteoclast differentiation or activation (Edgar et al., 2003; Kajimura et al., 2011). Jin et al. (2017) showed that chronic social defeat stress increases the plasma levels of RANKL, OPG, and TNF-α and decreases BMD in mice. However, CMS increases the gut level of serotonin and the bone expression of 5-HT_{1b}R but decreases the bone level of cAMP (Choi et al., 2022). cAMP level and bone mass are higher and gut serotonin is lower in 5-HT_{1b}R-deficient mice than in conventional mice (Li et al., 2019).

Consistent with the present study, our previous studies showed that n-3 PUFA supplementation not only reduces depressive behavior including anhedonia and immobility measured by SPT and FST but also increases bone mass in depressed rats exposed to stress (Choi et al., 2022; Jin et al., 2017). The supplementation of n-3 PUFA decreases the blood level of NTx and increases the bone levels of calcium in CMS-exposed rats (Choi et al., 2022). The supplementation of n-3 PUFA

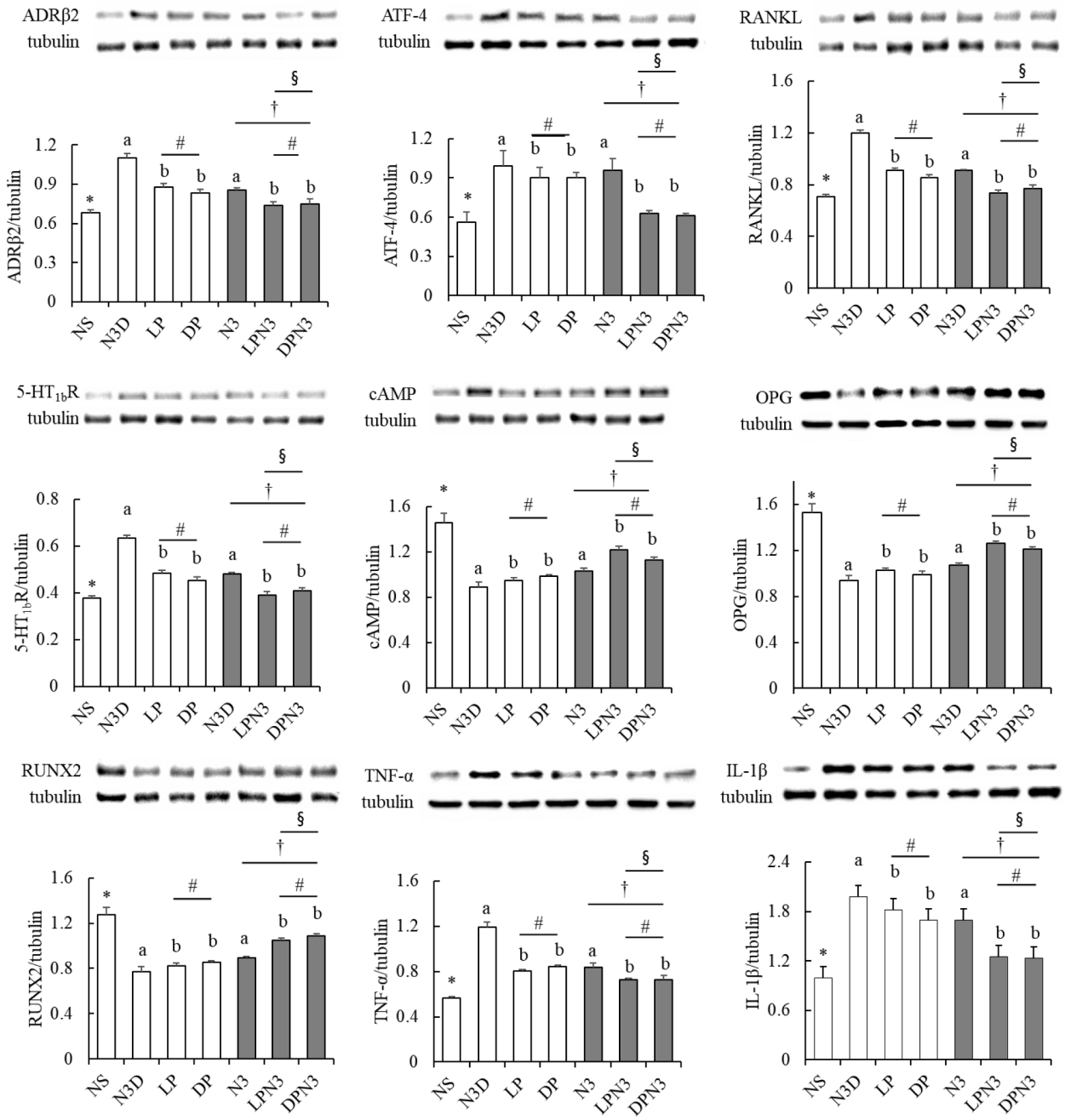

Fig. 4. Protein expression of bone-related markers NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; DPN3, n-3 PUFA diet and dead probiotics with CMS (n = 8); ADRβ2, beta 2 adrenergic receptor; ATF-4, activating transcription factor 4; RANKL, receptor activator of nuclear factor kappa-B ligand; 5-HT_{1b}R, 5-hydroxytryptamine receptor 1B; cAMP, cyclic adenosine monophosphate; OPG, osteoprotegerin; RUNX2, runt-related factor 2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β. *Values are significantly different between NS and N3D. #Values are significantly different between probiotics and nonprobiotics; a-bValues with different letters are significantly different between live and dead probiotics within the same diet; †Values are significantly different between n-3 PUFA and n-3 PUFA deficient diets; §Values show the significant synergistic effect of n-3 PUFA diet and probiotics.

also increases the expression of brain-derived serotonin and decreases the blood levels of CORT and ACTH, which are HPA axis-related hormones in rats exposed to CMS (Choi et al., 2021; Choi & Park, 2017; Jin & Park, 2015; Kim et al., 2020). Furthermore, n-3 PUFA ameliorates the CMS-induced bone loss by increasing the bone expression levels of OPG, RUNX2, and ADRβ2 and decreasing the bone expression levels of ATF4 and RANKL (Choi et al., 2022). Inflammation induces bone loss, but n-3 PUFA supplementation downregulates the expression of inflammatory cytokines in the bones of rats with OVX and apical periodontitis (Azuma et al., 2018; Jin et al., 2017). The supplementation of n-3 PUFA also decreases the gut levels of inflammatory cytokines and increases the bone mass of the femur of OVX rats (Zhu et al., 2021). Moreover, supplementation of EPA and DHA, and their derivatives upregulate anti-inflammatory mediators, while downregulate pro-inflammatory mediators, and ameliorate respiratory diseases and liver steatosis (Valenzuela et al., 2020; Zúñiga-Hernández et al., 2022). Ramos et al. (2005) reported that n-3 PUFA supplementation decreases the expression of 5-HT_{1b}R in the brain of tumor-bearing rats, suggesting that n-3 PUFA

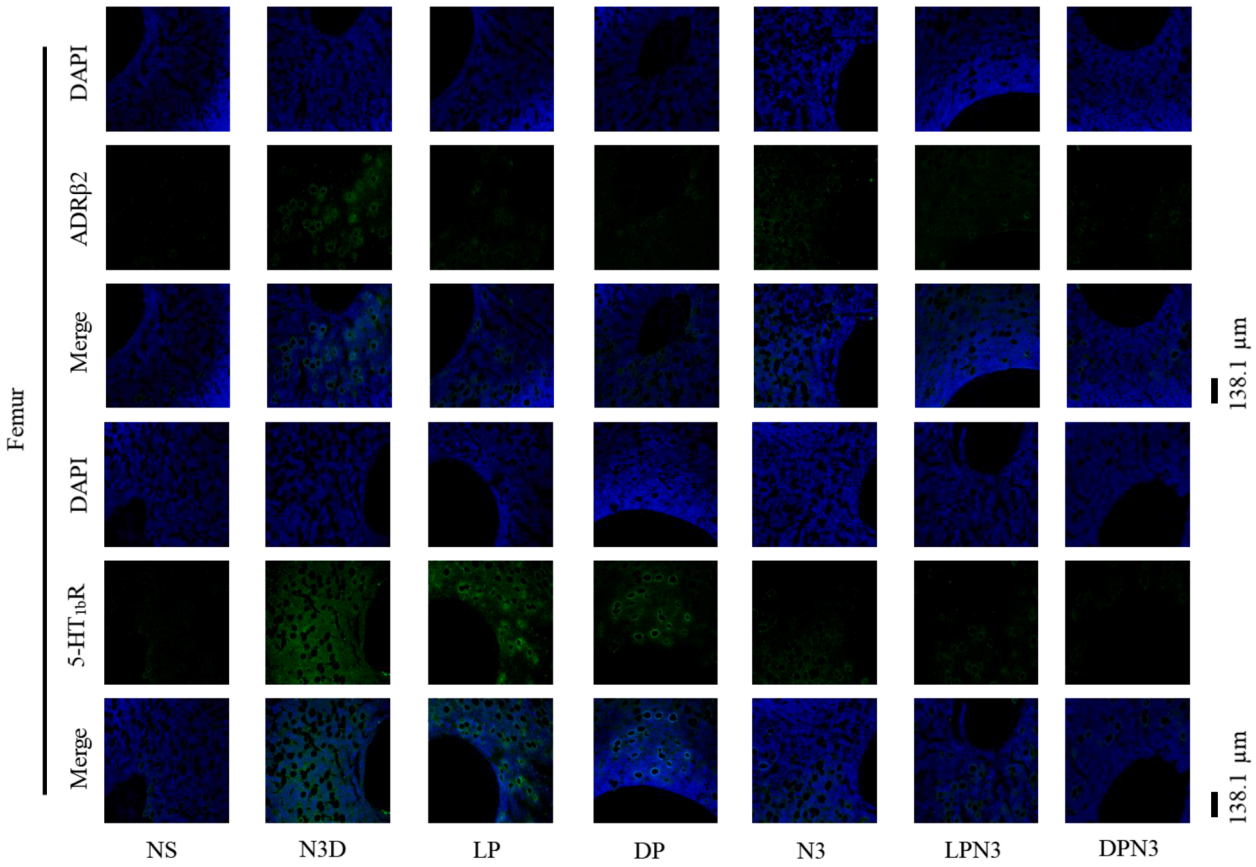

Fig. 5. Immunofluorescence staining of beta 2 adrenergic receptor (ADRβ2) and serotonin 1B receptor (5-HT_{1b}R) in the femur. ADRβ2 and 5-HT_{1b}R expression levels were visualized with DyLight® 488 (green), and DNA was stained blue (DAPI). Each staining of ADRβ2 and 5-HT_{1b}R was merged with DAPI, and images were shown. Scale bar = 138.1 μm. NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; DPN3, n-3 PUFA diet and dead probiotics with CMS.

stimulates serotonin release (Pimentel et al., 2013).

Consistent with the present study, a previous study demonstrated that the supplementation of live probiotics improves the depressive behavior measured by SPT and FST in rats with CMS (Li et al., 2019). However, the effect of probiotics on bones has not been investigated in stressed animal models, but the supplementation of live probiotics increases the BMC and BMD, biomarkers used to assess fragility-fracture risk, in the femur and the blood level of calcium in nonstressed rats (Narva, Collin, et al., 2004). In postmenopausal Japanese women, the supplementation of live probiotics also decreases the blood level of NTx (Takimoto et al., 2018). Furthermore, the supplementation of live probiotics increases alveolar bone mass by reducing the serum levels of inflammatory cytokines in OVX rats (Jia et al., 2021), suggesting that live probiotics affect the gut–bone axis. Previous studies showed that the supplementation of live probiotics increases the serotonin levels in the frontal cortex and hippocampus, while decreases the serotonin level in the gut, suggesting the interaction of brain-gut axis though serotonin (Li et al., 2019). Supplementation of live probiotics decreases the blood levels of CORT and ACTH (Liang et al., 2015) in rats with chronic stress. Moreover, the supplementation of live probiotics decreases the expression of RANKL in the bone marrow and spleen of osteoporotic mice (Dar et al., 2018) and increases the OPG expression in the femur of OVX mice (Ohlsson et al., 2014). Consistent with the present study, a previous study indicated that the supplementation of live probiotics ameliorate bone loss by increasing the bone level of cAMP and decreasing the bone expression of 5-HT_{1b}R (Heath & Hen, 1995).

Previous studies suggested that the strains used in the present study, namely, *Bifidobacterium longum*, *Lactobacillus helveticus*, and *Lactobacillus plantarum*, not only have bone-protecting effects but also have antidepressant effects (Collins et al., 2017; Li et al., 2018). Parvaneh et al. (2015) reported that the supplementation of *Bifidobacterium longum* increases serum osteocalcin (a bone formation marker), decreases serum C-terminal telopeptide (a bone resorption marker), alters the microstructure of the femur, and consequently increases the BMD of OVX rats. The supplementation of *Lactobacillus helveticus* also increases the BMC and BMD of the femur of growing rats (Narva, Collin, et al., 2004) and the content of tartrate resistant acidic phosphatase, a bone formation marker of bone marrow cells obtained from mice (Narva, Halleen, et al., 2004). In addition, *Lactobacillus plantarum* supplementation ameliorates bone loss by promoting osteoblast differentiation and inhibiting RANKL-induced osteoclast differentiation in OVX mice (Yang et al., 2020) and increases femoral BMD in OVX mice (Chiang & Pan, 2011). Li et al. (2018) reported that treatment with multistrain probiotics, including *Bifidobacterium longum*, *Lactobacillus helveticus*, and *Lactobacillus plantarum* decreases the inflammatory cytokines of the hippocampus of CMS mice.

Although previous studies mainly focused on live probiotics, few studies have compared the efficiency of live and dead probiotics (Liao et al., 2019; Liu et al., 2016; Moraes et al., 2020; Wei et al., 2019). Along with the present study, Moraes et al. (2020) reported that live and dead probiotics reduce the mesial alveolar bone loss in rats with ligature-induced periodontitis. Wei et al. (2019) showed that live and dead probiotics increase the hippocampal expression of brain-derived neurotropic factor and glucocorticoid receptors in chronic CORT-treated mice. Moreover, Liao et al. (2019) showed that live and dead probiotics decrease the serum levels of CORT and inflammatory

Table 2
Long chain fatty acid compositions of bone phospholipids[1]

Fatty acids (%)	NS	CMS						*P*-value[2]			
		N3D	LP	DP	N3	LPN3	DPN3	CMS	Diet	Probiotics	Diet × Probiotics
14:0	0.20 ± 0.04	0.20 ± 0.09	0.19 ± 0.05	0.19 ± 0.05	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.03	0.949	0.758	0.164	0.178
16:0	25.54 ± 0.68	24.98 ± 0.69	25.06 ± 0.75	25.05 ± 0.64	24.98 ± 0.72	25.00 ± 0.78	25.10 ± 0.65	0.121	0.977	0.928	0.978
18:0	23.26 ± 1.66	23.12 ± 1.39	23.06 ± 1.98	23.02 ± 1.05	23.65 ± 1.65	23.54 ± 2.11	23.29 ± 1.25	0.858	0.366	0.922	0.971
SFA	49.83 ± 0.96	49.52 ± 0.68	49.56 ± 1.20	49.50 ± 0.40	50.16 ± 0.91	50.06 ± 1.29	49.93 ± 0.59	0.480	0.052	0.914	0.947
18:1n9	20.74 ± 0.38	21.01 ± 0.38	20.85 ± 0.37	20.79 ± 0.01	21.03 ± 0.37	21.07 ± 0.43	21.07 ± 0.45	0.177	0.112	0.791	0.586
MUFA	21.61 ± 0.39	21.88 ± 0.40	21.73 ± 0.39	21.67 ± 0.02	21.91 ± 0.39	21.95 ± 0.45	21.96 ± 0.47	0.200	0.117	0.809	0.625
18:2n6	8.69 ± 0.01	8.68 ± 0.01	8.68 ± 0.01	8.68 ± 0.01	8.65 ± 0.02	8.65 ± 0.09	8.64 ± 0.01	0.098	<0.001	0.749	0.555
20:4n6	12.50 ± 0.24	12.44 ± 0.19	12.58 ± 0.24	12.61 ± 0.08	12.07 ± 0.20	12.00 ± 0.41	12.06 ± 0.01	0.542	<0.001	0.600	0.338
22:4n6	2.30 ± 0.04	2.31 ± 0.04	2.33 ± 0.05	2.34 ± 0.02	2.23 ± 0.04	2.22 ± 0.09	2.22 ± 0.02	0.693	<0.001	0.904	0.430
22:5n6	1.91 ± 0.02	1.91 ± 0.01	1.91 ± 0.02	1.91 ± 0.08	0.20 ± 0.02	0.20 ± 0.04	0.21 ± 0.01	0.232	<0.001	0.268	0.734
n-6 PUFA	26.14 ± 0.32	26.08 ± 0.26	26.26 ± 0.33	26.30 ± 0.12	23.88 ± 0.28	23.79 ± 0.54	23.84 ± 0.01	0.671	<0.001	0.689	0.372
20:5n3	0.34 ± 0.07	0.35 ± 0.01	0.35 ± 0.04	0.35 ± 0.07	0.38 ± 0.06	0.37 ± 0.08†§	0.372 ± 0.02†§	0.618	<0.001	0.291	0.020
22:5n3	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.04	0.12 ± 0.02	0.43 ± 0.07	0.42 ± 0.09	0.43 ± 0.03	0.837	<0.001	0.527	0.663
22:6n3	1.47 ± 0.23	1.57 ± 0.01	1.50 ± 0.47	1.59 ± 0.23	2.77 ± 0.22	2.94 ± 0.28	3.00 ± 0.09	0.240	<0.001	0.392	0.364
n-3 PUFA	2.08 ± 0.24	2.19 ± 0.01	2.12 ± 0.48	2.21 ± 0.25	3.72 ± 0.23	3.88 ± 0.30	3.95 ± 0.10	0.251	<0.001	0.441	0.451

*Values are significantly different between NS and N3D.
†Values are significantly different between n-3 PUFA diet and n-3 PUFA deficient diet.
$^{\#}$Values are significantly different between probiotics and nonprobiotics.
$^{a\text{-}b}$Values with different letters are significantly different between probiotics within the same diet.
§Values show the significant synergistic effect of n-3 PUFA diet and probiotic.
[1] Values are expressed as mean and standard deviation; NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS.
[2] Independent *t*-test was used to determine the CMS effect; diet effect was determined via two-way ANOVA, and probiotic effect was determined through two-way ANOVA followed by Duncan's post hoc test.

Table 3
Levels of SCFAs in the gut.[1]

	NS	CMS						*P*-value[2]			
		N3D	LP	DP	N3	LPN3	DPN3	CMS	Diet	Probiotics	Diet× Probiotics
Acetic acid (μM/g)	157.28 ± 7.19	132.86 ± 5.44^{a}	148.27 ± 4.56$^{\#b}$	146.87 ± 6.79$^{\#b}$	144.34 ± 3.10$^{\dagger a}$	148.79 ± 3.59$^{\#\dagger\S b}$	147.65 ± 5.42$^{\#\dagger\S b}$	<0.001	0.009	<0.001	0.008
Propionic acid (μM/g)	81.01 ± 0.68	71.04 ± 0.70^{a}	72.30 ± 0.05$^{\#b}$	72.30 ± 0.04^{b}	71.45 ± 0.15$^{\dagger a}$	79.25 ± 0.30^{§,b}	79.24 ± 0.17$^{\#\dagger\S b}$	<0.001	<0.001	<0.001	<0.001
Isobutyric acid (μM/g)	18.40 ± 0.21	17.25 ± 0.06	16.41 ± 1.37	16.85 ± 1.10	17.13 ± 1.03	16.70 ± 0.79	17.13 ± 1.21	<0.001	0.637	0.262	0.833
Butyric acid (μM/g)	77.16 ± 0.47	61.47 ± 0.93^{a}	65.16 ± 0.55$^{\#b}$	64.72 ± 1.16$^{\#b}$	62.36 ± 1.13$^{\dagger a}$	74.30 ± 0.63$^{\#\dagger\S b}$	74.40 ± 0.54$^{\#\dagger\S b}$	<0.001	<0.001	<0.001	<0.001

$^{a\text{-}b}$Values with different letters are significantly different between probiotics within the same diet.
§Values show the significant synergistic effect of n-3 PUFA diet and probiotic.
* Values are significantly different between NS and N3D.
†Values are significantly different between n-3 PUFA and n-3 PUFA deficient diets.
$^{\#}$Values are significantly different between probiotics and nonprobiotics.
[1] Values are expressed as mean and standard deviation; NS, n-3 PUFA deficient diet, and water with no stress; N3D, n-3 PUFA deficient diet and water with CMS; LP, n-3 PUFA deficient diet and live probiotics with CMS; DP, n-3 PUFA deficient diet and dead probiotics with CMS; N3, n-3 PUFA diet and water with CMS; LPN3, n-3 PUFA diet and live probiotics with CMS; and DPN3, n-3 PUFA diet and dead probiotics with CMS.
[2] Independent *t*-test was used to determine the CMS effect; diet effect was determined via two-way ANOVA, and probiotic effect was determined through two-way ANOVA followed by Duncan's post hoc test.

cytokines in a maternal separation mouse model. Liu et al. (2016) observed that live but not dead probiotics improve anxiety-like behaviors and increase brain-derived serotonin and dopamine levels in germ-free mice. The supplementation of live and dead probiotics increases fecal metabolites and decreases cytokine production in the spleen of conventional mice, but only live probiotics increase fecal metabolites and decrease cytokine production in germ-free mice; these results suggest that dead probiotics are ineffective in germ-free mice (Sugahara et al., 2017).

In line with previous studies, the present study showed that n-3 PUFA and probiotics supplementations modulated the inflammatory cytokines including IL-1β and TNF-α. Dogan et al. (2022) studied the synergic effects of n-3 PUFA and live probiotics and demonstrated that their combination improves alveolar bone loss and decreases the serum levels of cytokines, including IL-1β, IL-10, and IL-6, compared with those of the single use of n-3 PUFA and live probiotics in rats. The combination treatments of n-3 PUFA and probiotics synergistically decrease serum cytokine levels in obese rats (Kobyliak et al., 2017; Kobyliak, Falalyeyeva, et al., 2018) and patients with nonalcoholic fatty liver disease (Kobyliak, Abenavoli, et al., 2018) compared with those of the single use of probiotics. Additionally, supplementation of n-3 PUFA increases the abundance of SCFA-producing bacteria and levels of SCFA (Nogal et al., 2021), and modulates energy expenditure by regulating brown adipose tissue activity and white adipose tissue browning (Zapata et al., 2022). Robertson et al. (2017) showed that the supplementation of n-3 PUFA increases the abundance of Lactobacillus in conventional mice, suggesting that n-3 PUFA could be a prebiotic. In the present study, n-3 PUFA supplementation also increased the abundance of Lactobacillus and *Blautia* (Fig. S2). Rodrigues et al. (2012) reported that the supplementation of probiotics and yacon flour, well-known prebiotics, synergistically increases the calcium concentration in the tibia and the femoral fracture strength compared with those of their single use in rats. Furthermore, the bone-protecting effect of probiotics is partly attributed to gut microbiota modulation, thereby increasing the expression of estrogen receptors in OVX mice (Chen et al., 2021). Our previous study also reported that the supplementation of n-3 PUFA increases the bone mass and bone expression of estrogen receptor-α in OVX rats (Jin et al., 2017). Therefore, probiotics and n-3 PUFA can exert a synergistic bone-protective effect through the modification of estrogen receptors.

5. Conclusions

This study has few limitations. First, the bone-protecting mechanism of n-3 PUFA and probiotics was not confirmed in HTR2B gene knockout model. Second, this study measured bone loss only in the femur and tibia, not in other sites, although the femur can be the optimal site for reflecting osteoporosis (Singh et al., 1970). The single use of n-3 PUFA, live probiotics, or dead probiotics ameliorated bone loss induced by CMS. To our knowledge, this study was the first to investigate the synergistic bone-protective effects of n-3 PUFA and probiotics in CMS rats. However, further studies are needed to confirm the synergistic bone-protecting effects of n-3 PUFA and probiotics in patients with depression.

CRediT authorship contribution statement

Yunjung Lee: Methodology, Investigation, Formal analysis, Writing – original draft. **Haemin Oh:** Methodology, Formal analysis, Writing – review & editing. **Miyea Jo:** Methodology, Formal analysis, Writing – review & editing. **Hyunji Cho:** Methodology, Formal analysis, Writing – review & editing. **Yongsoon Park:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Funding

This work was supported by a grant from the National Research Foundation of Korea funded by the Korea government (MSIT; NRF-2021R1A2B02002208). We would like to thank Essayreview (www.essayreview.co.kr) for English language editing.

The study involving animal care and experiments was complied with the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-21-0092).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105363.

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