



Synergistic Antidepressant-like Effects of Biotics and n-3 Polyunsaturated Fatty Acids on Dopaminergic Pathway through the Brain-Gut Axis in Rats Exposed to Chronic Mild Stress

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Abstract

Probiotics, postbiotics, and n-3 polyunsaturated fatty acids (PUFA) have antidepressant-like effects. However, the underlying mechanisms of the dopaminergic pathway are unclear. The present study investigated the hypothesis that probiotics and postbiotics combined with n-3 PUFA synergistically improve depression by modulating the dopaminergic pathway through the brain-gut axis. Rats were randomly divided into seven groups: non-chronic mild stress (CMS) with n-6 PUFA, and CMS with n-6 PUFA, n-3 PUFA, probiotics, postbiotics, probiotics combined with n-3 PUFA, and postbiotics combined with n-3 PUFA. Probiotics, postbiotics, and n-3 PUFA improved depressive behaviors, decreased blood concentrations of interferon- γ , and interleukin-1 β , and increased the brain and gut concentrations of short chain fatty acids and dopamine. Moreover, probiotics, postbiotics, and n-3 PUFA increased the brain and gut expression of glucocorticoid receptor and tyrosine hydroxylase; brain expression of l-type amino acid transporter 1 and dopamine receptor (DR) D1; and gut expression of DRD2. The expression of phosphorylated protein kinase A/protein kinase A and phosphorylated cAMP response element-binding protein/cAMP response element-binding protein increased in the brain, however, decreased in the gut by the supplementation of probiotics, postbiotics, and n-3 PUFA. There was synergistic effect of probiotics and postbiotics combined with n-3 PUFA on the depressive behaviors and dopaminergic pathway in blood, brain, and gut. Moreover, no significant difference in the dopaminergic pathways between the probiotics and postbiotics was observed. In conclusion, probiotics and postbiotics, combined with n-3 PUFA have synergistic antidepressant-like effects on the dopaminergic pathway through the brain-gut axis in rats exposed to CMS.

Keywords Brain-gut axis · Chronic mild stress · Dopaminergic pathway · N-3 PUFA · Postbiotics · Probiotics

Introduction

Depression is increasing and leads to suicide, which is the fourth major cause of death worldwide [1]. Depression downregulates the serotonergic pathway [2], which in turn modulates the dopaminergic pathway [3]. A decrease in the concentration of dopamine (DA) was observed in the brain of depressed rodents exposed to chronic mild stress (CMS) [4–6], and in the gut of depressed mice exposed to cold stress [7].

Our previous studies reported that supplementation of biotics including probiotics and postbiotics, and n-3 polyunsaturated fatty acids (PUFA) had antidepressant-like effects by modulating the serotonergic pathway in the brain and gut of rats exposed to CMS [8, 9]. Administration of n-3 PUFA increased the concentration of DA and the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of DA, in the brain of depressed mice exposed to the forced swimming test (FST) [10, 11]. Moreover, administration of probiotics increased the concentration of DA and the expression of TH, dopamine receptor, and protein kinase A (PKA) in the brain of depressed rodents induced by obesity [12], and exposed to CMS [13, 14], social frustration stress [15], and restraint stress [16]. Administration of postbiotics also increased the DA concentration in the brain of depressed mice treated with corticosterone (CORT) [17] and *Salmonella* [18]. Since

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previous studies suggested that both probiotics and postbiotics modified gut microbiome composition and metabolites such as short chain fatty acids (SCFA), probiotics and postbiotics could have similar effects [19]. However, the effects of probiotics, postbiotics, and n-3 PUFA on the brain and gut dopaminergic pathway have not been studied yet.

Our previous studies have shown that supplementation of probiotics and postbiotics combined with n-3 PUFA had a synergistic effect on the serotonergic pathway in the brain and gut of depressed rats exposed to CMS [8, 9]. Since n-3 PUFA, as prebiotics, modified fecal level of SCFA and gut microbiome, n-3 PUFA could have synbiotic effect with probiotics or postbiotics [8]. Valent et al. [20] also showed that supplementation of probiotics combined with n-3 PUFA significantly increased the brain concentration of total DA, compared to probiotics alone in obese mice. However, the synergistic effect of biotics combined with n-3 PUFA on the dopaminergic pathway in the brain and gut has not yet been investigated. Therefore, this study aimed to investigate the hypothesis that supplementation of biotics combined with n-3 PUFA has a synergistic antidepressant-like effect on the dopaminergic pathway through the brain-gut axis in rats exposed to CMS. Additionally, we hypothesized that probiotics and postbiotics would have similar effects on the dopaminergic pathway in the brain and gut of depressed rats exposed to CMS.

Materials and Methods

Animals, and Diet and Oral Supplementation

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Hanyang University (HY-IACUC-21-0092). The Wistar rats (Central Lab. Animal Inc., Seoul, Korea) were housed in a ventilated air-conditioned room maintained at 22 ± 1 °C with a 12 h light/dark cycle and 40–50% humidity. The experimental diets were isocaloric modified American Institute of Nutrition-93G diet containing 16% fat of the total energy intake (E%) with either 0 E% (n-6 PUFA diet) or 1 E% eicosapentaenoic acid (EPA) and DHA in a ratio of 6:4 (n-3 PUFA diet; Supplementary Table 1). The n-6 PUFA and n-3 PUFA diets were made up of grape seed oil (Sajo Haepyo, Seoul, Korea) with 0 g and 7.33 g of re-esterified triglyceride fish oil (Epax, Ålesund, Norway), respectively. The biotics consisted of *Bifidobacterium longum*, *Lactobacillus helveticus*, and *Lactobacillus plantarum* in a 1:1:1 ratio (Lactomason, Jinju, Korea), and to prepare postbiotics, the probiotics were heat killed. Sterile water (1 mL), with or without 6×10^9 colony-forming units/day of biotics was administered orally.

Experimental Design

One week after acclimatization, five-week-old rats were supplemented with experimental diet and oral supplementation for 12 weeks (Supplementary Fig. 1A). Animals were randomly divided into seven groups ($n = 6$ per group): n-6 PUFA diet without CMS (NS), n-6 PUFA diet with CMS (N6), n-6 PUFA diet and probiotics with CMS (Pro), n-6 PUFA diet and postbiotics with CMS (Post), n-3 PUFA diet with CMS (N3), n-3 PUFA diet and probiotics with CMS (N3Pro), and n-3 PUFA diet and postbiotics with CMS (N3Post). After six weeks of supplementation, rats were exposed to CMS for five weeks, as described in a previous study [21]. The procedure of CMS included eight different mild stresses with a weekly rotation plan: food deprivation, water deprivation, cage tilting (45°), soiled cage (300 mL of 25 ± 1 °C water), group housing, no bedding, stroboscopic illumination (130 flashes/min), and intermittent illumination every 2 h (Supplementary Fig. 1B). The rats in the NS group were housed separately from those in the CMS groups to avoid CMS disturbances. After CMS, a pre-sucrose preference test (SPT) was performed for four days, and the SPT was performed the following day. The pre-FST and FST were respectively performed the day before and on the day of euthanasia.

Behavior Test

During the pre-SPT, rats were provided with a bottle of sterile water and 1% sucrose solution. The bottles were weighed daily and switched on every day to avoid side bias. Rats were deprived of water and food for 10 h before the SPT, and then provided with a bottle of sucrose solution and a bottle of sterile water with food for 12 h. The sucrose preference index (%) was calculated as follows: (grams of sucrose solution intake/total grams of liquid intake) \times 100.

During the pre-FST and FST, rats were individually forced to swim inside a cylinder (height, 50 cm and diameter, 20 cm) containing 25 ± 1 °C water up to a height of 30 cm. The pre-FST and FST were performed for 15 and 5 min, respectively, and videotaped. The duration of immobility, swimming, and climbing were measured by three raters who were blinded to the test conditions.

Blood and Tissue Collection

The rats were anesthetized with Zoletil and Rompun (30 mg/kg and 10 mg/kg body weight, respectively). Serum was collected, and the dissected brain (hippocampus) and gut (colon) were rinsed with saline, weighed, and immediately

frozen in liquid nitrogen. Blood and tissue samples were then stored at $-80\text{ }^{\circ}\text{C}$ for further analysis.

Gas Chromatography

The brain and gut (100–300 mg) were homogenized, total lipids were extracted, and phospholipids were separated using thin-layer chromatography (SIL G-25; Machereynagel GmbH & Co, Duren, Germany) [22]. Phospholipids were methylated, and the composition of long-chain fatty acids (14:0 or more) was analyzed using gas chromatography (Shimadzu 2010 AF; Shimadzu Scientific Instrument, Tokyo, Japan) with a $100\text{ m} \times 0.25\text{ mm}$ inner diameter and $0.20\text{ }\mu\text{m}$ film capillary column (SP2560; Supelco, Bellefonte, PA, USA) [23]. The carrier gas was hydrogen at a flow rate of 40 mL/min , and the injection and detection temperatures were $230\text{ }^{\circ}\text{C}$ and $240\text{ }^{\circ}\text{C}$, respectively. The run temperature began at $180\text{ }^{\circ}\text{C}$, and increased by $5\text{ }^{\circ}\text{C/min}$ to $200\text{ }^{\circ}\text{C}$ and by $10\text{ }^{\circ}\text{C/min}$ to $240\text{ }^{\circ}\text{C}$. The split ratio was 10:1, and external standards (GLC-OQA; Nu-Check Prep Elysian, MN, USA) were used to identify the fatty acids. The coefficient of variation was 4.3%.

To calculate the concentration of the SCFA, an internal standard (7:0; Cat # 75190, Sigma-Aldrich, Burlington, MA, USA) was added during homogenization of the brain and gut tissues, and NaOH and phosphoric acid were added for stabilization and acidification [24]. The SCFA, including acetic (2:0), propionic (3:0), isobutyric (4:0*i*), and butyric acids (4:0*n*), were analyzed using gas chromatography (Shimadzu 2010 AF; Shimadzu Scientific Instrument, Tokyo, Japan) with a $15\text{ m} \times 0.53\text{ mm}$ inner diameter and $0.50\text{ }\mu\text{m}$ film capillary column (25326; Supelco, Bellefonte, PA, USA). Nitrogen was used as the carrier gas at a flow rate of 40 mL/min , and the injection and detection temperatures were set at $190\text{ }^{\circ}\text{C}$. The run temperature was set at $80\text{ }^{\circ}\text{C}$ and increased by $5\text{ }^{\circ}\text{C/min}$ up to $190\text{ }^{\circ}\text{C}$, and the split ratio was 10:1. External standards (WSFA-4 mixture, Matreya, State College, PA, USA) were used to identify the fatty acids.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum concentrations of interleukin (IL)- 1β and interferon (INF)- γ were measured using an ELISA kit (Cat # LS-F5627; LS-F5066-1, LSBio, Seattle, WA, USA). To measure DA concentration, the brain and gut were homogenized, and the supernatants were analyzed using an ELISA kit (Cat # ENZ-KIT188-0001, Enzo Life Science, Farmingdale, NY, USA). All measurements were performed in triplicates and quantified using standards on a spectrophotometer (Multiscan GO, Thermo Scientific, Waltham, MA, USA), according to the manufacturer's protocol.

Western Blot Analysis

The brain and gut were homogenized and centrifuged at $10,000 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$, and protein content was measured using a bicinchoninic acid assay (Cat # 23225, Pierce Biotechnology, Rockford, IL, USA). Protein ($30\text{ }\mu\text{g}$) was separated on 10% polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and blocked for 1–1.5 h at room temperature with 5% skim milk or bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST). The membrane was incubated with primary antibodies against the TH (Cat # LS-C60884; brain, 1:1000; gut, 1:250), dopamine receptor (DR) D1 (Cat # LS-C332275; brain, 1:2000; gut, 1:1000), DRD2 (Cat # LS-C748000; 1:1000), PKA (Cat# LS-C63197; brain, 1:1000; gut, 1:500), phosphorylated PKA (pPKA; Cat # LS-C63197; brain, 1:1000; gut, 1:500), cAMP response element-binding protein (CREB; Cat # 9197; 1:1000), phosphorylated CREB (pCREB; Cat # 9198; 1:1000), glucocorticoid receptor (GR; Cat # 183127; 1:500), and l-type amino acid transporter1 (LAT1; Cat # LS-B15706; 1:1000) in 5% BSA in TBST overnight at $4\text{ }^{\circ}\text{C}$. Antibodies against CREB and pCREB were purchased from Cell Signaling Technology (Danvers, MA, USA), antibody against GR was purchased from Abcam (Cambridge, UK), and other antibodies were purchased from LSBio (Seattle, WA, USA). Membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies, anti-rabbit IgG (Cat # LS-C60884, LSBio, Seattle, WA, USA), with 5% BSA in TBST for 1 h at room temperature. Immunoreactive bands were visualized with the UV setting on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) to estimate the quantity of protein in each lane, and β -actin was used for normalization. Western blot analysis was performed in triplicates.

Immunofluorescence Staining

The brain (dentate gyrus region of the hippocampus) and gut (colon) samples were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, cut into $10\text{ }\mu\text{m}$ and $4\text{ }\mu\text{m}$ sections, respectively, and mounted on glass slides. The sections on the glass slides were then immersed in xylene followed by alcohol, rehydrated in wash buffer, and heated in sodium citrate buffer (0.01 M, pH 6.0). The sections were blocked with a PAP pen (Enzo Life Science, Farmingdale, NY, USA) and incubated with primary antibodies against the DRD1 (Cat # LS-C332275; 1:50; LSBio, Seattle, WA, USA) and DRD2 (Cat # sc-5303; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at $4\text{ }^{\circ}\text{C}$ overnight. The sections were incubated with goat anti-rabbit IgG H&L with Alexa Fluor[®] 488 (Cat # LS-C354919, LSBio, Seattle, WA, USA) and 555 (Cat # ab150078, Abcam, Cambridge, UK) for 2 h at room temperature, washed, and mounted with Fluoroshield

containing 4', 6-diamidino-2- phenylindole (DAPI; Cat # ENZ-53003-M010; Enzo Life Science, Farmingdale, NY, USA). The Leica TCS SP8 X confocal microscope and Leica AF imaging software (Leica Microsystems, Wetzlar, Germany) were used to evaluate immunofluorescence images.

Statistical Analysis

Data were analyzed using SPSS for Windows (version 27.0; SPSS Inc., Chicago, IL, USA). All values are expressed as mean with standard deviations and differences were considered significant at $P < 0.05$. Data were analyzed using independent t -tests with the CMS factor. The effects of the n-3 PUFA diet and biotics, and their synergistic effects were determined using two-way analysis of variance followed by Duncan's *post hoc* test.

Results

Depressive Behaviors, DA, and Inflammatory Cytokines

The sucrose preference, the duration of climbing and swimming, and the DA concentration in brain and gut were significantly decreased by CMS, however, the duration of immobility was increased by CMS (Table 1). Probiotics, postbiotics and n-3 PUFA significantly increased the sucrose preference, climbing duration, and the DA concentration in brain and gut. There were no significant differences in depressive behaviors and DA concentration between the probiotics and postbiotics. Supplementation of biotics combined with n-3 PUFA had a significant synergistic effect on the climbing duration and DA concentration in brain and gut. Moreover, no significant effects of diet and biotics on dietary intake, body weight, or organ weight were observed, however, CMS significantly decreased the final body weight (Supplementary Table 2).

The concentrations of IL-1 β and INF- γ in the blood were significantly ($P < 0.001$) increased by the CMS, but decreased by the supplementation of probiotics, postbiotics, and n-3 PUFA (Fig. 1). No significant difference in the cytokines between probiotics and postbiotics was observed. Supplementation of biotics combined with n-3 PUFA had a significant ($P < 0.001$) synergistic effect on the concentrations of inflammatory cytokines in the blood.

Fatty Acid Composition of Brain and Gut

The concentrations of 2:0 (brain, $P = 0.035$; gut, $P < 0.001$), 3:0 (brain, $P = 0.036$; gut, $P < 0.001$), 4:0i (brain, $P = 0.005$, gut, $P < 0.001$), and 4:0n ($P < 0.001$) were decreased by CMS (Fig. 2). The concentrations of 2:0 (brain, $P = 0.036$;

gut, $P = 0.043$), 3:0 (brain, $P = 0.037$; gut, $P < 0.001$), and 4:0n ($P < 0.001$) were increased by the supplementation of n-3 PUFA. Probiotics and postbiotics also increased the concentrations of 2:0 (brain, $P = 0.044$; gut, $P < 0.001$), 3:0 (brain, $P = 0.011$; gut, $P < 0.001$), and 4:0n ($P < 0.001$). There were no significant differences between probiotics and postbiotics on the concentrations of SCFA in brain and gut. Supplementation of biotics combined with n-3 PUFA had significant synergistic effects on the concentrations of 2:0 (brain, $P = 0.029$; gut, $P = 0.011$), 3:0 (brain, $P = 0.006$; gut, $P < 0.001$), and 4:0n (brain, $P = 0.003$; gut, $P < 0.001$).

Biotics and CMS had no significant effect on the phospholipid composition of long-chain fatty acids in the brain and gut (Supplementary Tables 3 and 4). Supplementation of n-3 PUFA significantly ($P < 0.001$) increased 20:5n3, 22:5n3, 22:6n3, and total n-3 PUFA, and decreased 18:2n6, 20:2n6, 20:3n6, 20:4n6, 22:4n6, 22:5n6, and total n-6 PUFA.

Dopaminergic Pathway in Brain and gut

Figure 3 showed that CMS decreased the brain expression of DRD1 ($P < 0.001$), DRD2 ($P < 0.001$), LAT1 ($P = 0.002$), TH ($P < 0.001$), pPKA/PKA ($P < 0.001$), and pCREB/CREB ($P < 0.001$). Probiotics, postbiotics, and n-3 PUFA increased the brain expression of DRD1 ($P < 0.001$), LAT1 ($P < 0.001$), TH ($P < 0.001$), pPKA/PKA (diet, $P = 0.002$; biotics, $P < 0.001$), and pCREB/CREB ($P < 0.001$). Supplementation of biotics, combined with n-3 PUFA had significant synergistic effects on the brain expression of DRD1 ($P < 0.001$), LAT1 ($P = 0.003$), TH ($P < 0.001$), pPKA/PKA ($P = 0.047$), and pCREB/CREB ($P = 0.031$). Figure 4 showed that CMS significantly ($P < 0.001$) decreased the expression of DRD2, GR, and TH, and increased the expression of pPKA/PKA and pCREB/CREB in gut. Probiotics, postbiotics, and n-3 PUFA significantly ($P < 0.001$) increased the expression of DRD2, GR, and TH and decreased the expression of pPKA/PKA and pCREB/CREB in gut. Supplementation of biotics, combined with n-3 PUFA had significant synergistic effects on the gut expression of DRD2 ($P = 0.004$), TH ($P = 0.012$), pPKA/PKA ($P = 0.042$), and pCREB/CREB ($P = 0.033$). Consistently, immunofluorescence staining showed that probiotics, postbiotics, and n-3 PUFA increased the expression of hippocampus dentate gyrus region DRD1 and colon DRD2 (Fig. 5). There were no differences between probiotics and postbiotics on the expression of the dopaminergic pathway in brain and gut.

Discussion

The present study demonstrated that supplementation of probiotics and postbiotics, combined with n-3 PUFA exerted synergistic antidepressant-like effects by regulating the

Table 1 The effect of probiotics, postbiotics, and n-3 PUFA on depressive behaviors and dopamine in brain and gut

	NS		CMS						P-value														
			N6		Post		N3		N3Pro		N3Post												
					Pro		Post		N3		N3Pro		N3Post		Stress		Diet		Biotics		Diet × Biotics		
Depressive Behaviors																							
SI (%)	91.43 ± 1.93*	79.07 ± 2.06	80.68 ± 3.65‡	86.12 ± 2.23‡	81.93 ± 3.34†	86.79 ± 1.44‡,†	89.35 ± 0.72‡,†	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Immobility (s)	31.00 ± 1.41*	38.10 ± 3.58	37.15 ± 2.74	36.91 ± 2.16	35.12 ± 1.16†	34.05 ± 2.01†	32.74 ± 1.09†	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Climbing (s)	101.30 ± 3.39*	81.35 ± 1.56	82.49 ± 1.19‡	82.99 ± 1.00‡	82.80 ± 1.64†	86.04 ± 1.22‡,†,§	86.35 ± 1.21‡,†,§	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Swimming (s)	187.70 ± 4.46*	180.55 ± 3.76	180.36 ± 2.36	180.10 ± 1.43	182.08 ± 1.97	179.91 ± 1.24	180.91 ± 1.45	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dopamine (ng/mL)																							
Brain	150.32 ± 6.16*	66.71 ± 2.21	116.81 ± 4.63‡	123.61 ± 1.65‡	127.84 ± 2.52†	140.93 ± 1.91‡,†,§	130.64 ± 1.68‡,†,§	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Gut	42.89 ± 0.34*	40.25 ± 0.66	41.69 ± 0.38‡	41.19 ± 0.45‡	41.19 ± 0.47†	43.25 ± 0.63‡,†,§	43.40 ± 0.40‡,†,§	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means with standard deviation (n = 6/group); CMS, chronic mild stress; SI, sucrose preference index

NS, n-6 PUFA diet without CMS; N6, n-6 PUFA diet with CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

*P < 0.05 compared to N6

‡P < 0.05 compared to non-probiotic groups in the same diet with CMS

†P < 0.05 compared to the n-6 PUFA diet groups with CMS

§P < 0.05; Synergistic effects of biotics and n-3 PUFA

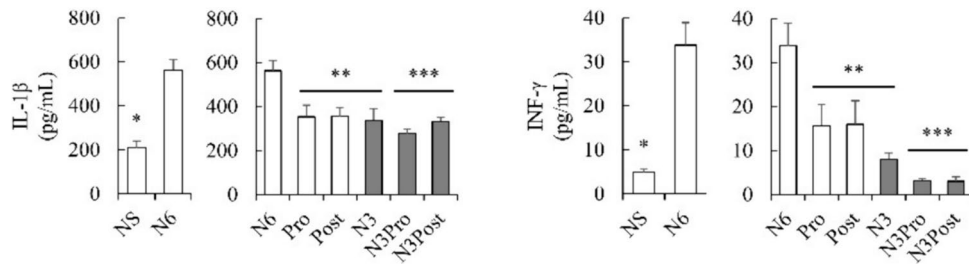
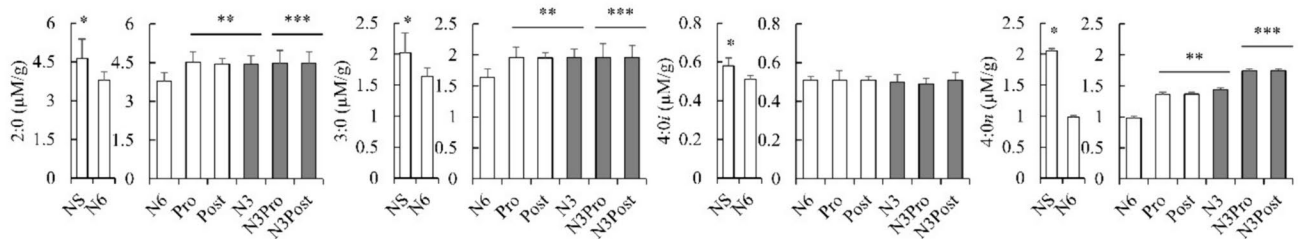


Fig. 1 Concentration of cytokines in blood. Values are mean with standard deviation ($n = 6/\text{group}$); *Values are significantly different between non-chronic mild stress (CMS) vs. CMS within the same diet; ** Values are significantly different between probiotics, postbiotics, and n-3 PUFA vs. n-6 PUFA diet within the same CMS condition; *** Values are significantly different between combination

of biotics and n-3 PUFA vs. probiotics, postbiotics, and n-3 PUFA within the same CMS condition; IL-1 β , interleukin-1 β ; INF- γ , interferon- γ ; NS, n-6 PUFA diet without CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

(A)



(B)

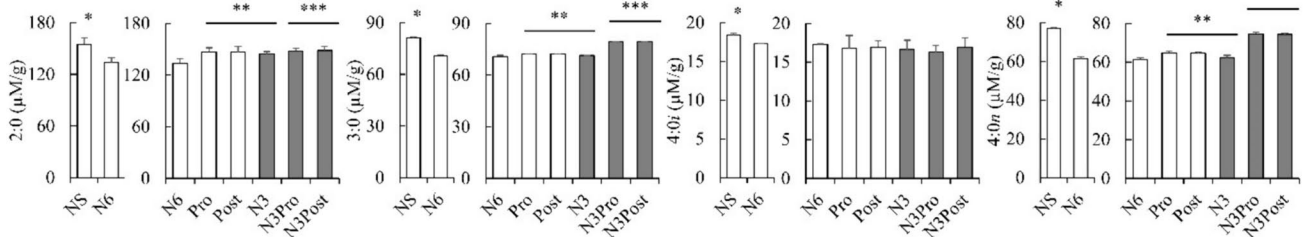


Fig. 2 Levels of short chain fatty acids in brain (A) and gut (B). Values are mean with standard deviation ($n = 6/\text{group}$); *Values are significantly different between non-chronic mild stress (CMS) vs. CMS within the same diet; ** Values are significantly different between probiotics, postbiotics, and n-3 PUFA vs. n-6 PUFA diet within the same CMS condition; *** Values are significantly different between

combination of biotics and n-3 PUFA vs. probiotics, postbiotics, and n-3 PUFA within the same CMS condition; NS, n-6 PUFA diet without CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

dopaminergic pathway through the brain-gut axis in rats exposed to CMS (Fig. 6). Alex et al. [3] showed that serotonergic pathway regulated dopaminergic pathway through serotonin receptor. Consistent with the present study, depression was modulated by the dopaminergic pathway in the brain through decreasing the concentrations of DA and SCFA, and the expression of TH, dopamine receptor, and PKA in depressed rodents exposed to CMS [4–6, 8, 9, 25,

26] and restraint stress [16]. Ohtsuki et al. [27] showed that the brain expression of LAT1 decreased in mice with Parkinson's disease (PD) induced by low concentration of DA. The present study also showed that brain expression of LAT1 decreased in depressed rats exposed to CMS. With respect to the gut dopaminergic pathway, the gut concentration of DA was also decreased in depressed mice exposed to cold stress [7], and the colonic expression of TH and dopamine

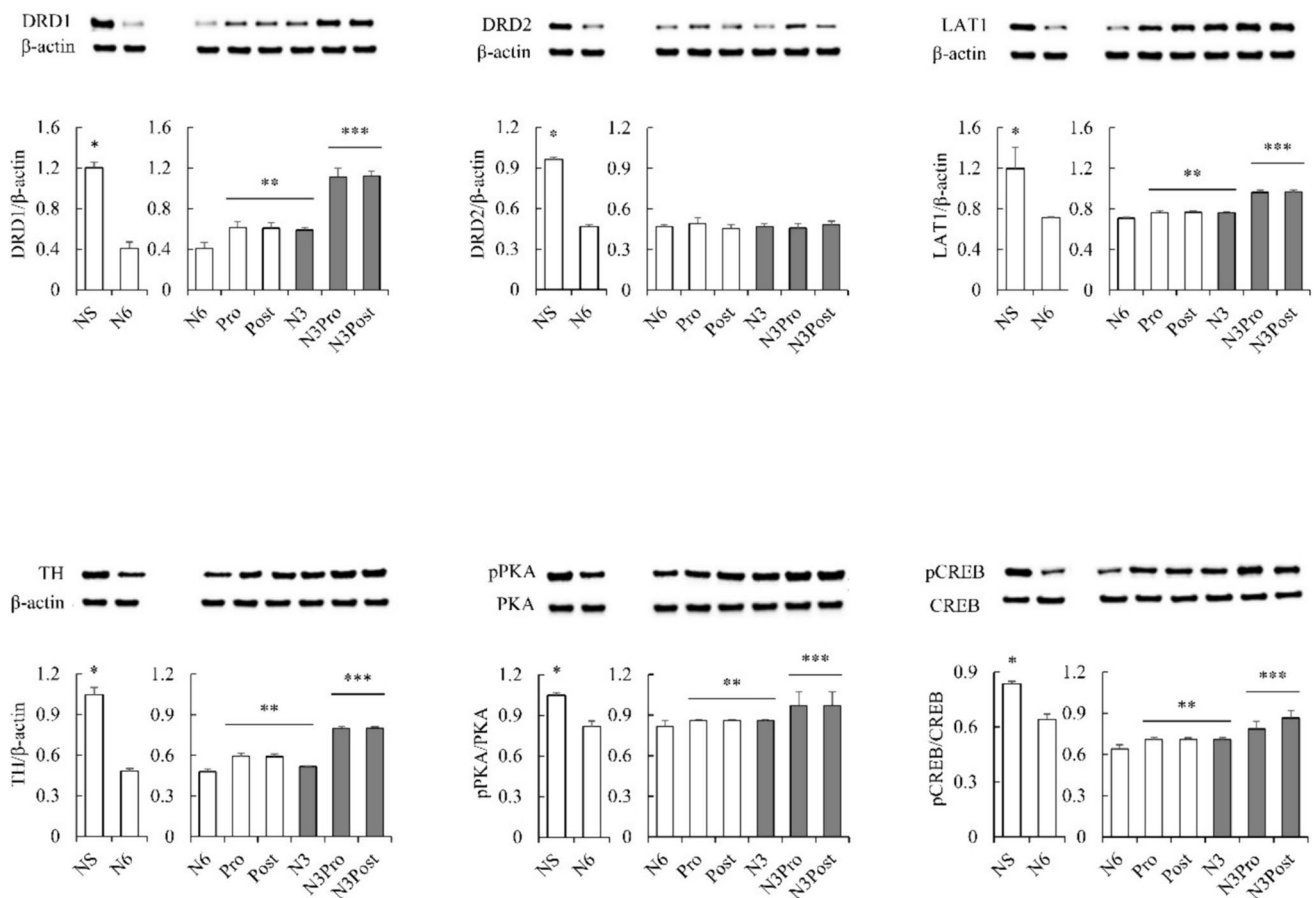


Fig. 3 The effect of probiotics, postbiotics, and n-3 PUFA on proteins related to dopaminergic pathway in brain. Values are mean with standard deviation ($n = 6$ /group); *Values are significantly different between non-chronic mild stress (CMS) vs. CMS within the same diet; **Values are significantly different between probiotics, postbiotics, and n-3 PUFA vs. n-6 PUFA diet within the same CMS condition; ***Values are significantly different between combination

of biotics and n-3 PUFA vs. probiotics, postbiotics, and n-3 PUFA within the same CMS condition; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; LAT1, l-type amino acid transporter 1; NS, n-6 PUFA diet without CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

receptors decreased in PD rats [28, 29]. Previous study showed that relative abundance of *Ruminococcaceae* in fecal was lower in depressed rat exposed to CMS [8], and PD patients [30], suggesting the relationship between dopaminergic pathway and gut microbiome. The present study is the first study to show that depression is modulated by the gut dopaminergic pathway in depressed rats exposed to CMS.

Consistent with the present study, previous studies have shown that n-3 PUFA and krill oil (rich in n-3 PUFA) increase the concentrations of DA and SCFA, and the expression of TH, and pCREB in the brain of depressed rodents exposed to CMS [8, 9, 31] and FST [11]. In the placenta of pregnant women, supplementation of n-3 PUFA during gestation and postpartum increased the gene expression of LAT1 compared to that in the control group [32]. Precursor of DA, L-3,4-dihydroxyphenylalanine (L-DOPA), is known to cross the blood brain barrier by LAT1 [33]. Metz et al. [34] showed that supplementation of fish oil normalized

the expression of DRD1 in the brain, which was impaired by amphetamine. Additionally, subcutaneous implantation of fluoxetine, a representative serotonin reuptake inhibitor (SSRI) increased DRD1 expression and serotonin concentration in the brain of depressed mice exposed to the FST [35]. As DA attaches to DRD1, the DRD1 is activated, enhancing the accumulation of cAMP levels and activating PKA [36]. Rashid et al. [37] showed that treatment of DHA metabolites increased the expression of pCREB/CREB and pPKA/PKA in neural stem cell treated with ethanol. The present study is the first study to demonstrate that n-3 PUFA increases the expression of LAT1, pPKA/PKA, and DRD1 in depressed animal model.

Our previous studies showed that n-3 PUFA increased the fecal abundance of *Ruminococcaceae*, the major butyric acid producer, and the concentration of SCFA, while decreasing the expression of pCREB in the gut of rats exposed to CMS [8, 9]. Patel et al. [38] reported that butyric acid increased

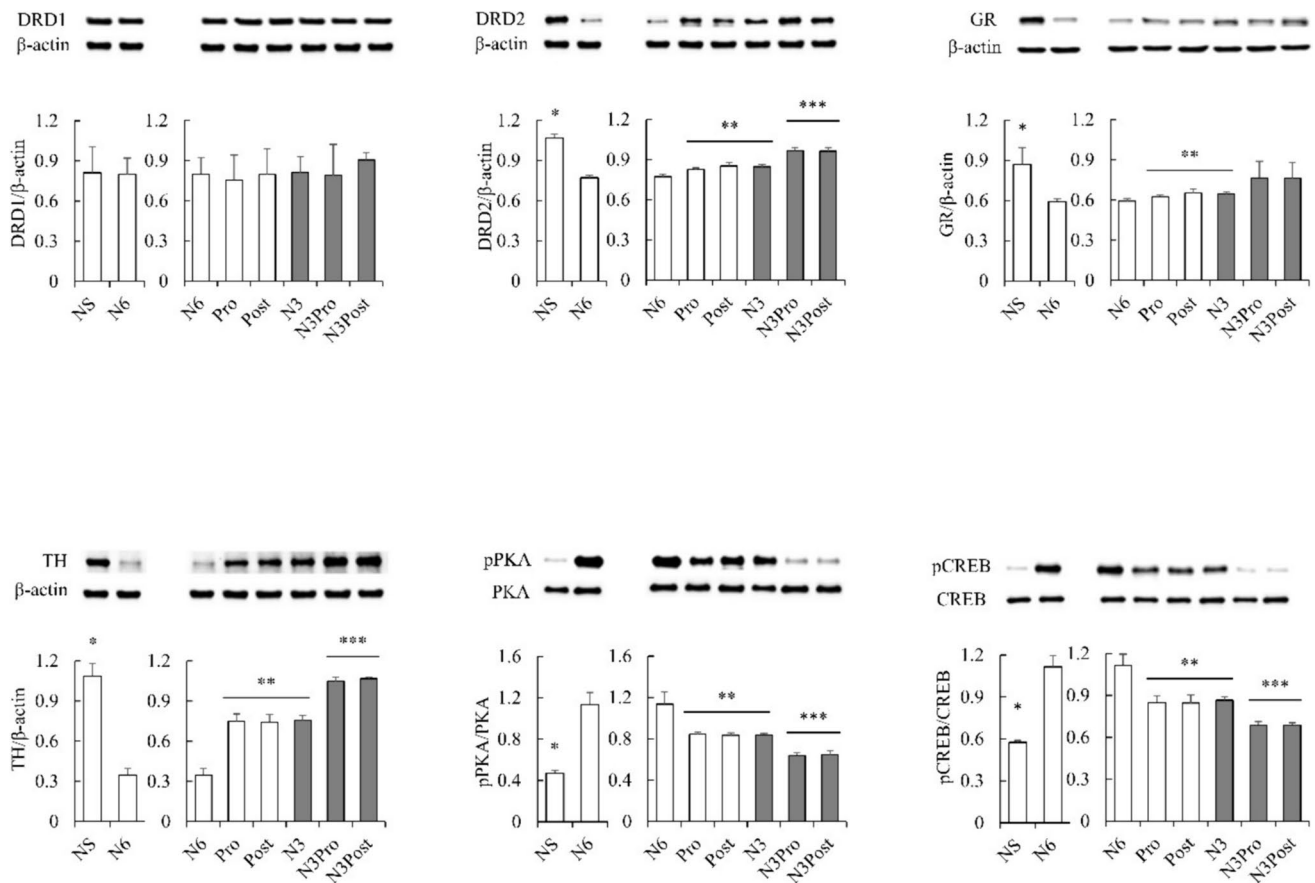


Fig. 4 The effect of probiotics, postbiotics, and n-3 PUFA on proteins related to dopaminergic pathway in gut. Values are mean with standard deviation ($n=6$ /group); *Values are significantly different between non-chronic mild stress (CMS) vs. CMS within the same diet; **Values are significantly different between probiotics, postbiotics, and n-3 PUFA vs. n-6 PUFA diet within the same CMS condition; ***Values are significantly different between combination of biotics and n-3 PUFA vs. probiotics, postbiotics, and n-3 PUFA

within the same CMS condition; CREB, cAMP response element-binding protein; GR, glucocorticoid receptor; pCREB, phosphorylated CREB; PKA, protein kinase A; pPKA, phosphorylated PKA; TH, tyrosine hydroxylase; NS, n-6 PUFA diet without CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

the transcription of TH by upregulating gene promoter in the adrenal gland organ cell. As supplementation of n-3 PUFA increased the gut concentration of butyric acid in depressed rats exposed to CMS [8, 9], administration of n-3 PUFA could also increase the expression of TH in the gut. Peng et al. [39] showed that the concentration of DA and the expression of TH decreased in the brain of colonic DRD2 knock out mice with PD compared with those of wild-type mice with PD. Rats with PD showed reduced DRD2 expression and dysmotility in the gut, suggesting the importance of gut DRD2 in the dopaminergic pathway [28]. Magro et al. [40] showed that treatment of $\text{INF-}\gamma$ decreased the L-dopa uptake in Caco-2 cell, and dopamine decarboxylase activity, the enzyme converting L-dopa to DA, increased concentration-dependently by L-dopa in colonic mucosa of colitis rat. Supplementation of fish oil decreased the concentration of $\text{INF-}\gamma$ in blood of mice with listeriosis [41], suggesting that

supplementation of n-3 PUFA could increase the concentration of DA in gut. The present study is the first study to demonstrate that n-3 PUFA modulates the gut dopaminergic pathway in depressed animal model by increasing the concentrations of DA and SCFA, and the expression of DRD2, and TH, and by decreasing the expression of pPKA/PKA and pCREB/CREB in the gut.

Consistent with the present study, our previous studies have shown that the administration of probiotics and postbiotics increase the concentration of SCFA and the expression of pCREB in the brain of depressed rats exposed to CMS [8, 9]. Supplementation of probiotics also increased the concentration of DA and the expression of TH, PKA, and DRD1 in the brain of depressed rodents induced by CMS [13], social frustration stress [15], restraint stress [16], and obesity [12]. Wu et al. [42] showed that treatment of probiotics increased the gene expression of LAT1 in intestinal cell exposed to

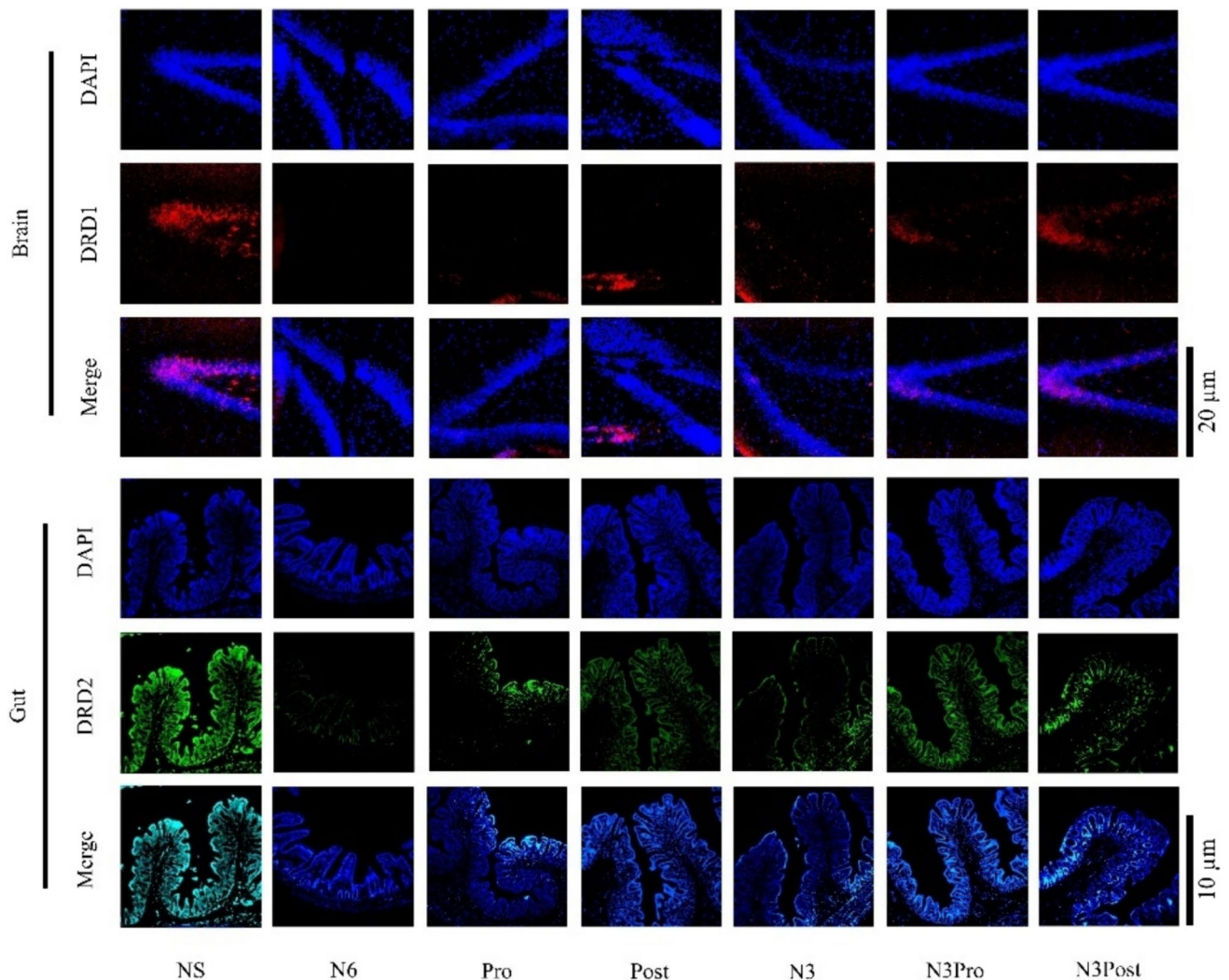


Fig. 5 Immunofluorescence staining of dopamine receptor D1 (DRD1) and D2 (DRD2) in hippocampus dentate gyrus region (brain) and colon (gut). Expression of DRD1 and DRD2 were visualized using Alexa Fluor 555 (red) and 488 (green), respectively, and DNA was stained blue (DAPI); Staining of DRD1, DRD2, and DAPI, and

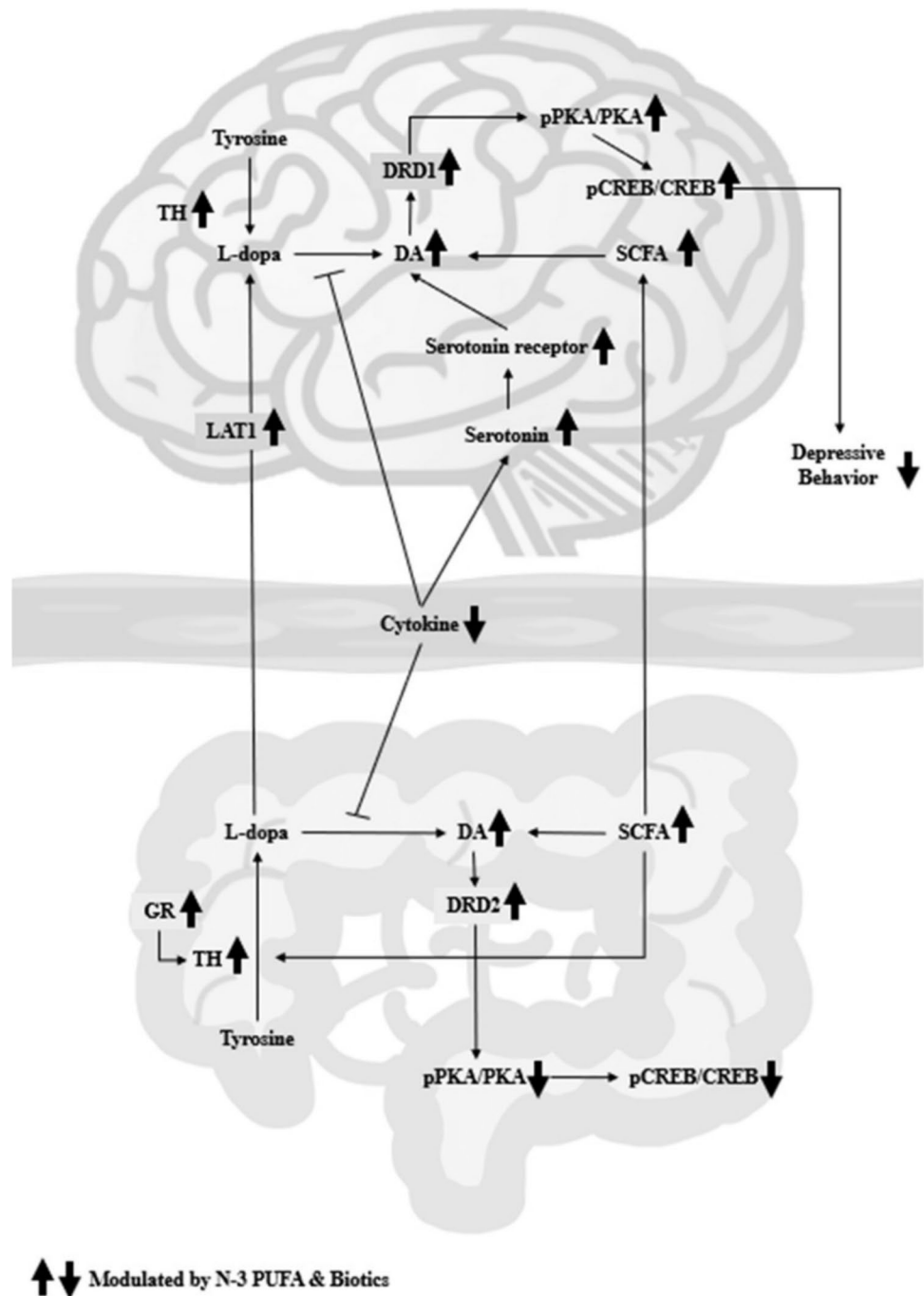
merged images are shown; NS, n-6 PUFA diet without CMS; Pro, n-6 PUFA diet and probiotics with CMS; Post, n-6 PUFA diet and postbiotics with CMS; N3, n-3 PUFA diet with CMS; N3Pro, n-3 PUFA diet and probiotics with CMS; N3Post, n-3 PUFA diet and postbiotics with CMS

oxidative stress. Consistent with the present study, supplementation of postbiotics increased DA concentration in the brain of depressed mice exposed to *Salmonella* [18] and treated with CORT [17]. There were no differences between probiotics and postbiotics on DA concentration in the brain of depressed mice exposed to *Salmonella* [18], suggesting that postbiotics could also modulate the brain dopaminergic pathway. The present study showed that probiotics and postbiotics have similar effects on the brain dopaminergic pathway in depressed animal model.

Our previous studies showed that administration of probiotics and postbiotics increased the fecal abundance of *Ruminococcaceae*, concentration of SCFA and the expression of GR, and decreased the expression of pCREB in the

gut of rats exposed to CMS [8]. It has been well-known that probiotics and postbiotics modified gut microbiome composition by microbiome itself and their metabolites [19]. Gut microbiome and their metabolites including SCFA, urolithin A, and taurine were associated with dopamine transporter and receptor in brain [43–45]. Additionally, GR-mediated glucocorticoid actions have known to sustain DA activation during stress via stimulation of TH in the cell body [46], suggesting that probiotics and postbiotics could increase the TH in gut. Cheon et al. [47] also showed that postbiotics increased the expression of TH, the rate-limiting enzyme of DA in human colon adenocarcinoma cell. As DA attaches to DRD2, the DRD2 is activated, inhibiting the production of cAMP levels and

Fig. 6 Abridged general view of the dopaminergic pathway for the antidepressant-like effect of probiotics, postbiotics, and n-3 PUFA. Probiotics, postbiotics, and n-3 PUFA increase the expression of the tyrosine hydroxylase (TH), leading to upregulation of the dopamine (DA) in the brain and gut; In addition, probiotics, postbiotics, and n-3 PUFA increase the gut levels of short chain fatty acids (SCFA) and decrease blood levels of inflammatory cytokines; SCFA can cross the brain-blood barrier and increase dopamine (DA) level in the brain, which attenuates depressive behavior; Probiotics, postbiotics, and n-3 PUFA increase the brain dopamine receptor 1 (DRD1) and gut dopamine receptor 2 (DRD2), which modify protein kinase A (PKA) signaling. CREB, cAMP response element-binding protein; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; GR, glucocorticoid receptor; LAT1, l-type amino acid transporter1; pCREB, phosphorylated CREB; PKA, protein kinase A; pPKA, phosphorylated PKA; TH, tyrosine hydroxylase



PKA [36]. Supplementation of probiotics also normalized the gene expression of PKA, which was impaired by constipation in the colon of mice [48]. The present study is the first study to demonstrate that probiotics and postbiotics modulate the gut dopaminergic pathway in depressed rats exposed to CMS. Supplementation of probiotics decreased the concentration of $\text{INF-}\gamma$ in brain of depressed mice exposed to CMS [49], suggesting that supplementation of probiotics could increase the concentration of DA in gut.

Our previous studies showed that supplementation of probiotics and postbiotics, combined with n-3 PUFA had synergistic effect on the hypothalamic-pituitary-adrenal axis and serotonergic pathway in the brain and gut of rats exposed to CMS [8, 9]. Valent et al. [20] also showed that supplementation of probiotics combined with n-3 PUFA significantly increased the concentration of total DA, including DA and its metabolites, compared to probiotics supplementation alone in obese mice. Our previous study suggested n-3

PUFA as a prebiotics by showing that supplementation of n-3 PUFA combined with probiotics and postbiotics elevated fecal abundance of *Ruminococcaceae* and gut concentration of butyric acid in depressed rats exposed to CMS [8]. Since postbiotics including fragments of microorganisms and metabolites, particularly SCFA had a beneficial effect to the host [19], the combination of postbiotics and n-3 PUFA could also exert a synergistic effect, which was comparable to those with probiotic and n-3 PUFA. Moreover, supplementation of probiotics combined with dietary fiber significantly decreased depression scores on the hospital anxiety and depression scale compared to probiotics supplementation alone in depressed hemodialysis patients [50]. Additionally, Zhao et al. [51] showed that fecal microbiota transplantation, as a synbiotics for the components of host gut microbiome, bacterial debris, and metabolic products, increased the brain expression of TH and improved gut dysbiosis in mice with PD.

To our knowledge, this is the first study to show antidepressant-like effects of probiotics, postbiotics, n-3 PUFA, and n-3 PUFA combined with probiotics and postbiotics on the dopaminergic pathway through the brain-gut axis. However, the present study has several limitations. First, synergistic antidepressant-like mechanisms of n-3 PUFA, probiotics, and postbiotics have not been demonstrated in DR knockout models. Second, Griffith et al. [52] showed that supplementation of α -linolenic acid, one of n-3 PUFA, improved anxiety behavior, and increased the concentration of DA in brain of diabetic mice, while the diet in the present study only contained EPA and DHA.

In conclusion, the present study suggests that probiotics, postbiotics, and n-3 PUFA exert a synergistic therapeutic effect on depression by modulating the dopaminergic pathway through the brain-gut axis. Further studies are required to confirm the antidepressant-like effects of probiotics, postbiotics, n-3 PUFA, and n-3 PUFA combined with probiotics and postbiotics on the dopaminergic pathway in patients with depression.

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Author Contributions Experiments and data analysis were performed by Hyunji Cho. The first draft of the manuscript was written by Hyunji Cho. Study design was supervised, and the manuscript was revised by Yongsoon Park. All the authors have read and approved the final manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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