Effects of Probiotic Bacteria on Central Neuronal Activation in Experimental Colitis

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ABSTRACT

Background: *Brain–gut axis dysregulation is observed in inflammatory bowel disease. However, the effect of altered gut flora on neuroimmunomodulation and its role in the pathogenesis of inflammatory bowel disease are unknown. The aims of this study are to determine (i) whether colitis modifies the expression of c-fos, a marker of general neuronal activation in the brain and (ii) whether this activation could be modulated by probiotic bacteria.*

Methods: *In this study, 28 Sprague–Dawley rats were divided into 4 groups: colitis-probiotic group, non-colitis-fed-control group receiving probiotic Lactobacillus delbrueckii subsp. Bulgaricus B3 strain for 7 days, colitis group, and sham group receiving only sodium chloride. Colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid–ethanol. The expression of c-fos was detected by immunohistochemistry in the brain tissue. Cytokines and inflammatory mediators were analyzed in the plasma. Histological scores and oxidative status were analyzed in the colon samples.*

Results: *The inflammatory response was accompanied by increased levels of cytokines, lipid peroxidation activities, c-fos expression in the medial nucleus of the amygdala, and decreased levels of antioxidant enzymes in the colitis ($P < .001$). Probiotic treatment reversed those effects. Also, histopathologic scores were significantly lower in the probiotic-treated groups compared to the colitis group ($P = .035$). In contrast, the expression of c-fos was significantly increased in the paraventricular nucleus of hypothalamus in the probiotic-treated rats ($P < .001$).*

Conclusion: *Colitis and intestinal inflammation are associated with the activation of neurons in the limbic system creating stress-like effects in the brain. Probiotics diversely modulate limbic response and hypothalamic axis activity in addition to protective effects in inflammation.*

Keywords: *C-fos, gut–brain axis, inflammatory bowel disease, lactobacillus, probiotic*

INTRODUCTION

Gut and the central nervous system (CNS) share a bidirectional signaling network known as the brain–gut axis. This network is very important physiologically to maintain homeostasis.[1,2] This bidirectional interaction involves CNS, sympathetic and parasympathetic nervous system, hypothalamic pituitary adrenal (HPA) axis, and enteric nervous system (ENS) which modulates intestinal functions, and vice versa. The gut microbiota is the key factor underpinning CNS signaling and is an active contributor to the homeostatic processes.[2,3]

The gut microbiota is a crucial component of human physiology which is modulated in coherence with host genome and responses to environmental factors (such as diet, stress). The inflammatory bowel diseases (IBD) appear to be the disorders of the host immune response to gut microbiota.[4,5] Therefore, modulation of microbiota with probiotics became popular as a treatment option in IBD. Inflammatory bowel diseases were reported in Western countries with a higher prevalence and incidence than in Asia. This encouraged studies involving the nutrition styles of Asian societies which have high consummation of probiotic products in daily life. Those studies have shown that probiotic products, primarily yogurt and kefir, have regulative effects on gut microbiota.[6-8] Recently meta-analyses showed evidence of the impact of probiotic treatment in IBD. In a meta-analysis of 23 randomized controlled trials covering 1763 adults, it

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has been shown that, with respect to placebo, probiotics increased rates of remission significantly in active ulcerative colitis patients.[9] Later on, in a meta-analysis of 27 randomized clinical trials measuring the effectiveness of probiotics in achieving remission in IBD patients, Lactobacillus probiotic mixture has a significant effect on ulcerative colitis patients and patients with Crohn's disease after surgery.[10]

Gut–brain axis dysregulation is observed in IBD. Psychological stress can cause IBD.[11] The IBD inflammation due to stress possibly occurs via HPA malfunctioning, modifications in microbiota, and alterations in enteric nervous system. In addition, variations in the bacterial composition of the gut lead to behavioral diversions and psychiatric comorbidity. Mental disorders were more likely to be seen in IBD patients.[12] However, in the IBD, the impact of dysbiosis (disruption of the gut microbiota) and bacterio-therapeutic efforts via probiotics on CNS function remains unclear. Furthermore, neuronal activation in response to intestinal inflammation and dysbiosis still needs to be investigated. Detection of c-Fos is used as an indicator of neuronal activation.[13,14]

The purpose of this study is to demonstrate (i) whether colitis modifies the presence of c-fos protein and (ii) whether this activation could be modulated by probiotic bacteria, or not.

MATERIALS AND METHODS

Animals

The study was approved by the Local Animal Ethics Committee. In this study, 28 female Sprague–Dawley rats (weight 150-200 g) were randomly divided into 4 groups; sham, fed-control, colitis, and probiotic groups. The rats were given water and rat chow ad libitum throughout the study.

Colitis Model

Under the xylazine (10 mg/kg, Rompum®, Bayer AG, Germany) and ketamine (60 mg/kg, Ketalar®, Pfizer Inc, USA) anesthesia, by intracolonic (8 cm from the anal orifice using a polyethylene cannula) administration of trinitrobenzene sulfonic acid (0.6 mL TNBS 5% w/v, Sigma Chemical Co., St. Louis, Oxoid, Ireland) with 0.25 mL ethanol (%50), colitis was induced. Sham rats received 0.9% NaCl instead of TNBS.

Probiotic Preparation

Probiotic was isolated from Turkish traditional natural yogurt. Early stationary phase cells of *Lactobacillus delbrueckii* subsp. *Bulgaricus* B3 strain were inoculated in MRS agar: De Man, Rogosa ve Sharpe agar plates and incubated at 37°C for 24-48 hours. Then, colonies were harvested with sterile loops in 0.5 mL sterile 0.9% NaCl. About 10^{12} to 10^{13} colony forming unit (CFU)/mL of bacteria were present in this solution prior to lyophilization. About 0.25 mL of cell suspension was then resuspended in 0.75 mL of sterile skim milk containing 10% (w/v) solids (Oxoid) in the vials. They were frozen at −80°C for one night, and they were freeze-dried with the freeze drier (Martin Christ Alpha 1-2) under vacuum (50 mTorr) for 24 hours. Then, cells were resuspended in a 1-mL sterile physiological saline solution. After that, they were serially diluted 10 times and 100-µL portions were spread plated in duplicate on MRS agar plates and incubated at 37°C. Viable bacteria count in each vial was adjusted as 10^7 to 10^8 CFU/mL.

Feeding Protocols

Probiotic groups received probiotic *L. delbrueckii* subsp. *Bulgaricus* B3 strain for 7 days. Probiotic (1 mL 10^7-10^8 CFU) was given by orogastric gavage daily to the control rats (fed-control group) and colitis-induced rats (probiotic group) for 7 days.

Sample Collection, Histopathology, and c-fos Immunohistochemistry

The rats were anesthetized with a high dose of ketamine at the end of experiment. The thorax and the abdomen were opened via a midline incision. Blood samples (2 mL) were taken through cardiac puncture. Descending aorta was ligated, the heads of the rats were perfused intracardially with of phosphate-buffered saline (PBS 0.1 M, pH 7.4, 50 mL) and followed by 4% paraformaldehyde (100 mL). Brains were removed. Subsequently, the whole colon was excised. To measure the tissue activities, colonic samples were taken from 15 cm (proximal) away from the anus. Half of the fresh tissues were kept at −80°C. The other half was fixed in 10% formalin, and routine histological processes were applied. Sections of 5 µm were cut and stained with hematoxylin and eosin and acidified toluidine blue (pH 2) for mast cells. They were counted in 10 sections via an eyepiece micrometer (OC-M, Olympus, Japan, X40).

Histological damage was evaluated according to scoring for chemically induced colitis.[15] Briefly, the scores represent the sum of scores from 0 to 2 for severity of inflammation, cell infiltration, crypt damage, focal ulceration, and goblet cell depletion. Histological assessment was made under the light microscope (Olympus BX 50 microscope).

After being embedded into paraffin, brain samples were 40 μm sectioned. Rat brain atlas coordinates were used for obtaining hypothalamic nuclei (–1.80 to 2.12 mm from bregma) and medial amygdaloid nucleus (–1.80 to 2.56 mm from bregma) sections.[16] Indirect immunohistochemistry was used for detection of c-fos protein detailed in Kilinc et al[14] C-fos protein presented neurons in the 6 sections were counted.

Analysis of Inflammatory Mediators in Serum and Oxidative Status of Colonic Tissue

Custom-made rat cytokine kits including interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) (Cytokine Rat 10-Plex Panel kit, Invitrogen, Massachusetts, USA) and kits including IL-8, IL-13, IL-17, matrix metalloproteinase (MMP)-2, MMP-3, MMP-9, nuclear factor kappa beta (NF-κB), macrophage inflammatory proteins (MIP) 1α and β, and intercellular adhesion molecule 1 (ICAM-1) (Rat ELISA Kit, Invitrogen) were used to measure the serum levels. The analyses were performed according to the manufacturers' recommendations.

Full wall sections of the colon were examined for oxidative stress. Myeloperoxidase (MPO) catalase, glutathione (GSH), glutathione reductase, glutathione disulfide, glutathione-*s*-transferase, superoxide dismutase (SOD), glutathione peroxidase (GPX), and malondialdehyde (MDA) levels were determined in the colon according to the method described previously.[17] The measurements are reported per mg^{-1} tissue wet weight.

Statistical Analysis

The data were expressed as the mean ± standard error and analyzed by SPSS (version 9.0; SPSS Inc., Chicago, IL, USA). Kolmogorov–Smirnov and Leven tests were used to confirm normality distribution. Differences among the groups were examined by analysis of variance, Kruskal–Wallis, and Mann–Whitney *U* tests. *P* value <.05 was considered statistically significant.

RESULTS

The weight difference between fed-control and sham groups was not significant. On the other hand, the colitis model group's weight loss was higher than the other groups throughout the whole experiment ($P < .016$, Figure 1). Probiotic treatment avoided excessive weight loss. Histopathological colitis score was significantly higher in the colitis group as compared to the probiotic groups (colitis vs probiotic: $P = .035$; colitis vs fed probiotic: $P = .007$, Figure 2).

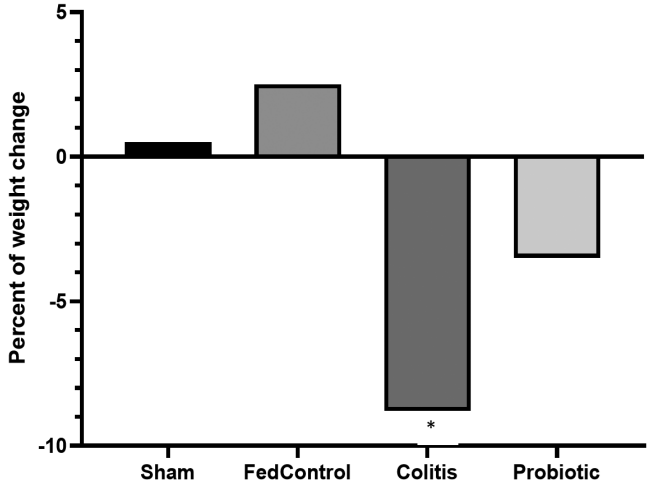

Figure 1. The graph of body weight percentage changes at the beginning and at the end of the study. The weight loss in the model colitis group was significantly higher than the other groups throughout the whole experiment ($P < .016$).

High level of cytokines (TNFα, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-8, IL-13, IL-17, and IFN-γ) in the colitis group indicate enhanced inflammatory response ($P < .001$, Table 1). Tumor necrosis factor alpha levels increased by 30 times and IL-6 levels by 15 times. The mentioned scores were significantly lower in the probiotic-treated colitis group. The cytokine levels of the fed-control group were similar to sham group with no significant difference.

Table 2 shows the antioxidant enzyme levels of study groups. Oxidative damage was observed in colitis group, with a high level of MDA (Figure 2, $P < .0003$) and MPO and reduced antioxidant enzyme activities (catalase, SOD, GSH, and GPX) ($P < .0001$, Table 2). In probiotic-treated group, antioxidant enzyme activities were higher, and MDA and MPO levels were lower than the colitis group ($P < .001$). The level of antioxidant enzyme in the fed-control group (probiotic-administered healthy rats) was not different from that of sham group.

Colitis significantly decreased the total mast cell count compared to the sham and the fed groups (colitis vs sham: $P = .032$, colitis vs probiotic $P = .048$) (Figure 3A and C). This reduction is most likely due to mast cell degranulation. The percentage of degranulation increased in the colitis group (Figure 3B). Probiotic treatment alleviated degranulation of mast cells (Figure 3C and D).

There is no significant difference between c-fos expression in fed-control and sham groups in any region of brain (Figure 4A and B). Colitis induced the expression

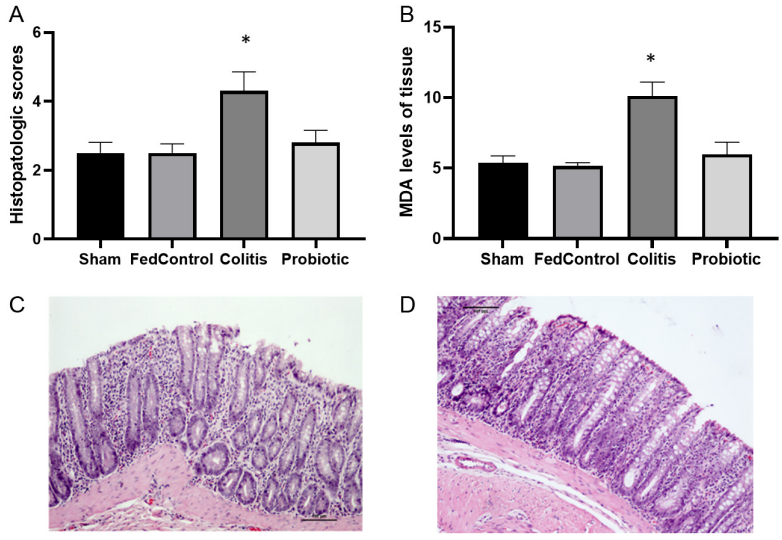

Figure 2. (A) The graph representing the histopathological scores of colonic damages in the groups. Histopathological colitis score was significantly higher in the colitis group compared to the probiotic groups (*colitis vs probiotic: $P = .035$; colitis vs fed probiotic: $P = .007$); (B) MDA levels of tissue in experimental groups. Results are expressed in mean ± SEM; n = 7. MDA levels were significantly higher in colitis group when compared to the other groups (*$P < .0003$) which prevented by probiotic treatment; (C) light micrographs of rat colonic mucosa (H&E, 10 × 10) in colitis group. Epithelial cell loss marked cell infiltration, edema, and dilated vessels; (D) light micrographs of rat colonic mucosa (H&E, 10 × 10) in the probiotic groups. Probiotic treatment prevents inflammation. H&E, hematoxylin and eosin; MDA, malondialdehyde.

Table 1. Inflammatory Mediators in Plasma

	Sham	Fed-Control	Colitis	Probiotic
TNF-α (pg/mL)	20.46 ± 2.11	20.86 ± 2.04	671.13 ± 105.31*	32.96 ± 7.01
IFN-γ (pg/mL)	24.11 ± 3.26	21.79 ± 3.04	62.15 ± 6.07*	25.97 ± 3.41
IL-1α (pg/mL)	5.70 ± 0.13	5.67 ± 0.31	21.83 ± 1.73*	6.04 ± 0.32
IL-1β (pg/mL)	8.61 ± 0.54	8.37 ± 0.73	15.35 ± 2.11*	8.71 ± 0.53
IL-2 (pg/mL)	8.92 ± 0.97	8.33 ± 0.85	32.97 ± 4.32*	8.82 ± 0.46
IL-4 (pg/mL)	41.35 ± 1.99	39.86 ± 3.17	70.70 ± 5.74*	47.49 ± 3.60
IL-6 (pg/mL)	5.70 ± 0.28	5.77 ± 0.13	894.87 ± 107.59*	12.58 ± 3.23
IL-8 (pg/mL)	88.57 ± 3.66	75.99 ± 3.25	209.41 ± 12.91*	83.68 ± 5.82
IL-10 (pg/mL)	159.20 ± 12.19	154.95 ± 11.00	2074.12 ± 242.61*	224.52 ± 42.17
IL-12 (pg/mL)	98.78 ± 8.27	87.93 ± 9.94	796.31 ± 9.53*	105.27 ± 11.88
IL-13 (pg/mL)	134.06 ± 5.73	124.01 ± 4.95	318.65 ± 22.05*	149.27 ± 11.20
IL-17 (pg/mL)	18.58 ± 1.05	20.68 ± 1.76	187.71 ± 21.45*	33.41 ± 5.18
NF-κB (pg/mL)	5.80 ± 0.23	5.52 ± 0.14	12.78 ± 0.48*	6.52 ± 0.12
ICAM-1 (pg/mL)	18.47 ± 1.21	21.57 ± 0.63	67.68 ± 1.97*	27.75 ± 1.23
MIP1α (pg/mL)	47.23 ± 1.02	51.07 ± 0.81	90.1 ± 2.02*	60.14 ± 1.07
MIP1β (pg/mL)	119.6 ± 3.35	123.4 ± 1.37	236.5 ± 5.11*	133.4 ± 1.85
MMP2 (ng/mL)	75.91 ± 1.17	69.09 ± 1.58	126.1 ± 1.11*	71.57 ± 1.2
MMP3 (ng/mL)	4.65 ± 0.06	5.32 ± 0.12	16.43 ± 0.28*	6.75 ± 0.23
MMP9 (ng/mL)	8.06 ± 0.21	7.75 ± 0.17	14.5 ± 0.52*	8.80 ± 0.11

Data are given as mean ± SEM. The severe inflammatory response was accompanied by a higher level of cytokines in the colitis group (*$P < .001$).
IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; ICAM-1, intercellular adhesion molecule 1.

Table 2. Oxidative Status of Colon Tissue in the Experimental Groups

	Sham	Fed-Control	Colitis	Probiotic
Catalase (nmol/mg)	6.95 ± 0.11	6.91 ± 0.08	3.86 ± 0.06*	6.83 ± 0.05
SOD (U/mg)	23.84 ± 0.50	24.31 ± 0.40	16 ± 0.64*	23.02 ± 0.66
GSH (nmol/mg)	68 ± 1.21	67.98 ± 1.11	38.19 ± 1.05*	67.02 ± 1.43
GSSG (nmol/mg)	6.82 ± 0.83	6.86 ± 0.06	12.1 ± 0.37*	7.11 ± 0.05
GST (U/mg)	3.92 ± 0.07	4.13 ± 0.05	1.87 ± 0.04*	3.96 ± 0.04
GR (nmol/mg)	40.23 ± 1.61	41.5 ± 1.42	25.7 ± 1.38*	40.28 ± 0.83
GPX (U/mg)	1.60 ± 0.03	1.57 ± 0.02	1.09 ± 0.04*	1.54 ± 0.03
MPO (U/mg protein)	0.59 ± 0.16	0.59 ± 0.03	1.31 ± 0.16*	0.64 ± 0.04

Data are given as mean ± SEM. All antioxidant enzyme activities were lower in the colitis group compared with those of sham, fed-control group and probiotic groups (*$P < .001$). SOD, superoxide dismutase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-*s*-transferase; GR, glutathione reductase; GPX, glutathione peroxidase; MPO, myeloperoxidase catalase.

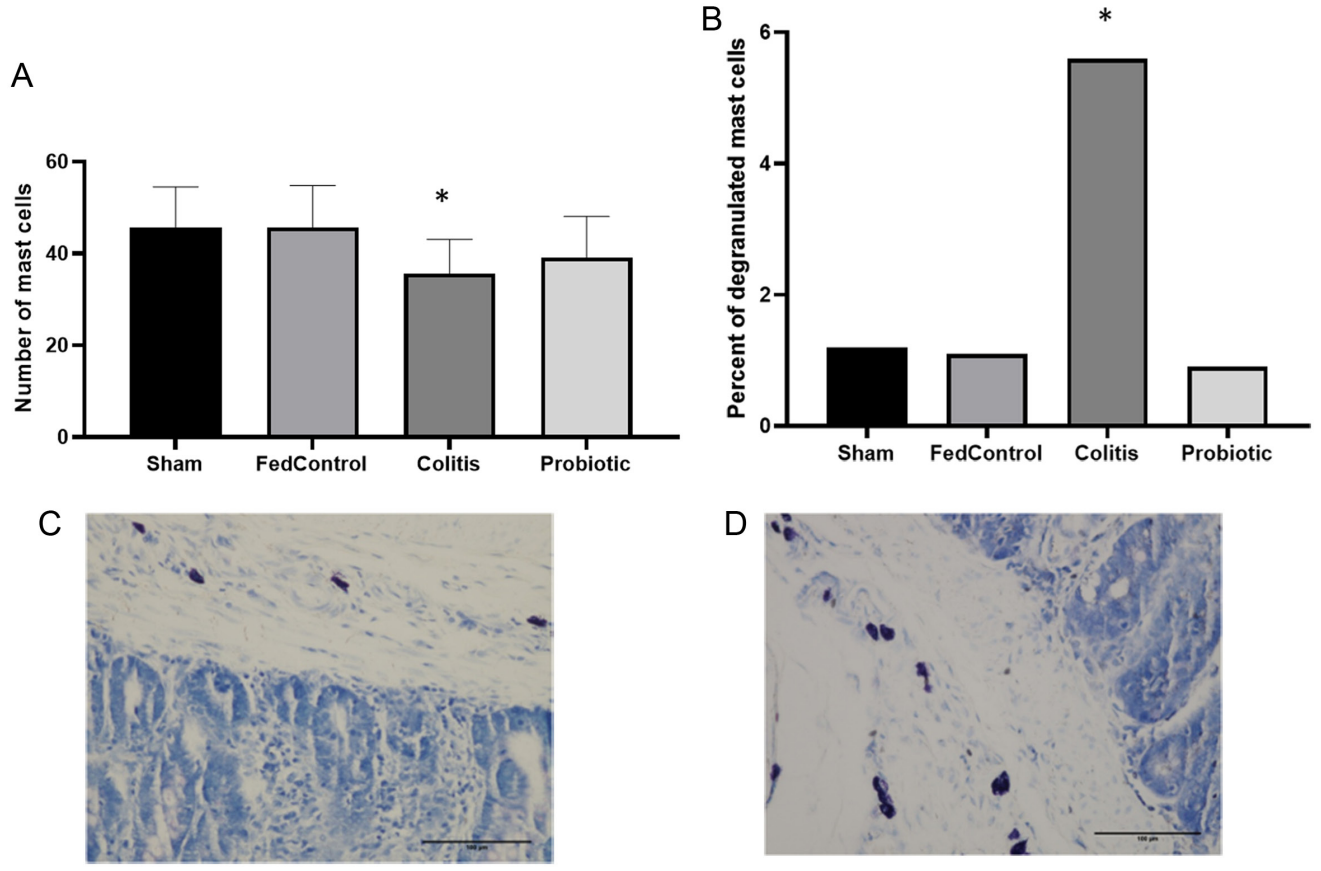

Figure 3. (A) Graphical representation of the mast cell counts in the groups. Colitis significantly decreased the total number of mast cells compared to the sham and the fed groups (*colitis vs sham: $P = .032$, colitis vs fed probiotic: $P = .048$); (B) percent of degranulated mast cells significantly increased colitis group, probiotic treatment prevented degranulation (*$P < .02$); (C) photomicrographs showing degranulated mast cells in the colitis group (toluidine blue, 20 × 10); (D) photomicrographs showing mostly granulated mast cells in the probiotic group (toluidine blue, 20 × 10).

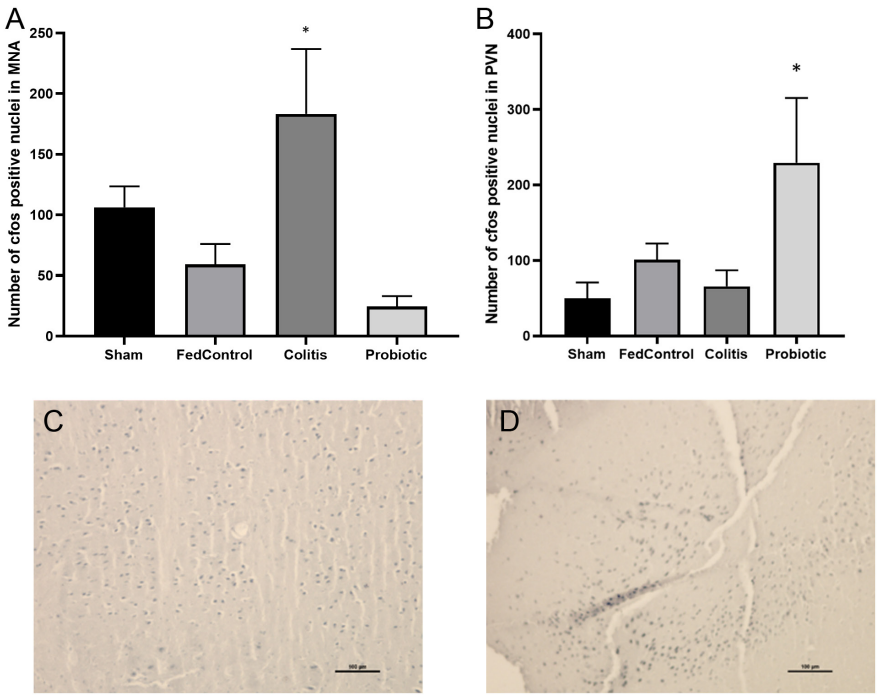

Figure 4. C-fos expression in the experimental groups. (A) The number of c-fos positive cells in MNA. Colitis significantly increased the expression of c-fos in this area as compared to the sham group (*$P < .001$). Probiotic treatment significantly reduced the c-fos expression in the MNA in comparison with the sham and colitis groups; (B) the number of c-fos positive cells in the PVN of the hypothalamus. The c-fos expression is significantly increased in this nucleus in the probiotic-treated groups with respect to the other groups (*$P < .001$); (C) photomicrographs showing c-fos positive cells (black) in the MNA of the colitis group (10 × 10); (D) photomicrographs showing c-fos positive cells (black) in the PVN of the probiotic group (10 × 10). MNA, medial nucleus amygdala; PVN, paraventricular nucleus.

of c-fos in the medial nucleus of the amygdala (MNA) (Figure 4A and C) ($P < .001$). Probiotic treatment significantly reduced the expression of c-fos in this area in comparison with colitis groups.

The c-fos expression in the paraventricular nucleus (PVN) of hypothalamus was not significant between colitis and sham groups (Figure 4B). In contrast, the c-fos expression was significantly increased in the PVN of the probiotic treated groups vis-à-vis the other groups ($P < .001$) (Figure 4B and D). Administration of probiotic did not induce neuronal activation in PVN in the healthy rats, while probiotic treatment increased it in colitis model rats.

DISCUSSION

The main conclusion of this study is that colitis-induced inflammation affects the amygdala, which is an essential component of the brain's limbic system, and this effect is attenuated by probiotic bacteria. In addition, probiotic bacteria may influence the function of HPA axis. This study demonstrates that CNS not only can detect intestinal inflammation and microbiota but also respond to them.

Inflammatory bowel disease is a chronic intestinal inflammatory condition. Neurological control of the intestine and the immune system can influence the clinical presentation and outcome of IBD. Dysfunction in the brain–gut bidirectional interaction can contribute to disease susceptibility. Microbiome within the gastrointestinal tract is the most important element of this communication.

Metabolic and genetic coding capacity of microbial population in the gastrointestinal tract may impact various aspects of the host physiology. Epigenetic mechanisms and mediators of genome–microbiome interactions may determine microbial sensory information that is encoded in the gut. Brain function is affected critically by host–microbe interactions, evolution, development, and behavior. Theoretically, gut microbiota or bacterial products can activate the afferent neurons or enteric nervous system, and this may cause neuroplastic changes in gut (peripheral sensitization). Host neural development and functions peripherally and centrally in the ENS is affected by the gut microbiota. In brain–gut bidirectional interactions, the amygdala is a key structure. The amygdala receives

information from the gut through 3 basic mechanisms: via vagus nerve afferent neurons, via immune cells and cytokines, and by enteroendocrine cells. Probably amygdala can distinguish bacteria which are non-pathogenic or potentially pathogenic even when inflammation is not present.

Dysbiosis-induced peripheral sensitization or alteration can modulate information transmission and brain central sensitization. Relying on the stimulus type, dysbiosis might falsely alert the limbic system which in turn can modify the sensory, motor, secretory, and immune functions of the gastrointestinal tract. This may increase the susceptibility to IBD development.

Gut inflammation and systemic inflammatory response may influence the gut–brain axis.[18,19] The concept of "inflammatory reflex" by which the CNS is able to detect inflammation was also supported in our study. Our results demonstrated that colitis induced c-fos activation of neurons in the MNA. Amygdala receives nociceptive information. Peripheral inflammation and inflammatory cytokines possibly activate amygdala, in response to colitis. This central signaling is conveyed via cytokine receptors and/or vagally mediated mechanisms or additional neural resources.[20,21]

Chemically induced colitis has been shown to cause mechanical and chemical hyperalgesia in the rodent gut.[22] In addition to inflammation, hyperalgesia may also cause amygdala activation.

In our study, on the other hand, amygdala activation was different in the presence of probiotic bacteria and intestinal inflammation. Adding probiotic treatment significantly reduced the c-fos expression in this area compared to the sham and colitis groups. Our results correspond to the study of Ait-Belgnaoui et al[13] which shows *Lactobacillus farciminis* treatment decreased visceral hyperalgesia induced expression of c-fos in the MNA of rats. Considering the information mentioned above, it can be speculated that probiotic bacteria are negatively correlated with the activity of amygdala (limbic system) which may represent the enteric nervous system. Thus, healthy bacteria affected nociceptive information modulation. Probably healthy flora or probiotic bacteria memorization and neuro-endocrine-immune axis within the gut do not alert limbic system or even reverse alerted amygdala activation due to inflammation.

Hypothalamic pituitary adrenal axis (HPA-axis) is involved in the regulation of stress response. Activation of the HPA axis is controlled by PVN of the hypothalamus. Neuronal activation induced by colon distension in this area has been observed.[13,23] This may be due to the difference between the colonic distension model for irritable bowel syndrome and the experimental colitis model for IBD. Possibly the perception of stress caused by distension is not the same of the inflammation. Even the severity of the inflammation, whether it is acute or chronic, or its duration might cause a difference. There was no significant difference in the expression of c-fos after colitis in the PVN of the hypothalamus compared to the sham group. Therefore, the activation of neurons at the PVN of hypothalamus in the probiotic-treated group is interesting. Moreover, the same activation is not observed in the fed-control group which are probiotic-administered healthy rats. Although the gut microbiota did not affect the basal activity of the HPA axis in colitis and fed-control rats, the response of the HPA axis to stress was altered in probiotic-treated colitis rats.

Unlike the limbic system, the HPA axis is responsible for the chronic adaptive responses as the core stress efferent. In this study, this differential c-fos activation may be related to the severity of colitis or duration of the inflammation. In addition, probiotic bacteria might influence stress response and regulates the set point for HPA activity.

More importantly, PVN of the hypothalamus and HPA axis appears to be susceptible not only to dysbiosis but also to beneficial microbial changes. Microbiota-related stress response attenuated the HPA axis activity. In addition to our results, previous findings[24] revealed that HPA axis activity differs according to the gut dysbiosis depending on the animal's species, strain, and sex, and also the type and/or duration of microbial content.

As a result, this study shows that the microbial content of the gut may play a key role in the functioning of the HPA axis. In addition, probiotic bacteria may modulate HPA axis activation in response to inflammation. In colitis, intestinal inflammation and peripheral cytokine response influence the CNS functions. Neuronal activation in response to intestinal inflammation in the amygdala is modulated by the probiotic bacteria in gut microbiota which additionally may alter attentional and motivational brain systems.

Probiotic treatment not only prevents inflammation, mast cell degranulation, and oxidative damage but also exerts protective effects via modulating the CNS in the brain–gut axis.

Ethics Committee Approval: The study was approved by the Abant İzzet Baysal University Animal Experiments Local Ethics Committee (No: B.30.2ABU.0.05.05.050.01.04-19).

Informed Consent: N/A.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept – N.S., F.T., G.U.; Design –N.S., F.T., S.I., T.F.; Supervision – N.S., F.T., G.U., B.A., A.K.; Resources – F.T., N.S., B.A., G.U.; Materials – F.T., N.S., B.A., A.K.; Data Collection – N.S., F.T., G.U., S.Y.C., T.F., A.K.; Analysis and Interpretation – N.S., F.T., G.U., T.F.; Literature Research – N.S., F.T.; Writing Manuscript – N.S., F.T.; Critical Review – N.S., F.T., G.U., S.Y.C.

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