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Comparison of the Effectiveness of Probiotics in the Experimental Colitis Model

Erhan KILICOGLU [1] *, Ayse Nur HAZAR-YAVUZ [1], Gul Sinemcan KABATAS [2], Yusufhan YAZIR [2], Levent KABASAKAL [1]

[1] Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul 34854, Türkiye.
[2] Stem Cell and Gene Therapies Research and Applied Center, Medical Faculty, Kocaeli University, Kocaeli 41380, Türkiye.
* Corresponding Author. E-mail: erhankilicoglumd@gmail.com. (E.K.); Tel. +90-0216-777 52 00.

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ABSTRACT: Inflammatory bowel disease (IBD) is a complex disease with an incompletely understood multifactorial etiopathogenesis. The importance of intestinal microbiota in IBD and the role of probiotics in the treatment are being extensively investigated. This study evaluated the therapeutic effects of *Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus reuteri*, *Saccharomyces boulardii* and *Bacillus clausii* in trinitrobenzene sulfonic acid (TNBS)-induced colitis animal model. Rats were randomly grouped into 7: control, colitis, colitis+BB-12, colitis+*L. reuteri*, colitis+*S. boulardii,* colitis+*B. clausii* and colitis+methylprednisolone (positive control) treatment groups. Colitis was induced by the administration of intracolonically 80 mg/kg TNBS. Treatments continued for 7 days. Body weight, stool consistency, rectal bleeding, and disease activity index (DAI) scores were evaluated and recorded daily. Macroscopic and microscopic colonic damage were evaluated and scored in the last day of experiment. Levels of malondialdehyde (MDA) and glutathione (GSH) and activity of myeloperoxidase (MPO) in the colonic tissues were detected by ELISA. Our results showed that treatments with BB-12, *L. reuteri*, *S. boulardii* and *B. clausii* significantly improved on clinical symptoms and decreased macroscopic and microscopic colonic damage on experimental colitis in rats. In addition, *L. reuteri* and *S. boulardii* significantly increased GSH levels and decreased MDA levels and MPO activity. When we evaluate our findings, we think that supplements of these specific probiotics may have beneficial effects in the treatment of IBD.

KEYWORDS: Probiotics; inflammatory bowel disease; experimental colitis; trinitrobenzene sulfonic acid; disease activity index.

1. INTRODUCTION

Inflammatory bowel disease (IBD) is represented by chronic inflammation of the gastrointestinal tract without infection (1). The etiopathogenesis of IBD is not perfectly understood. Genetic, environmental, microbial and immunological factors are all involved and play important roles in the pathogenesis of IBD (2). As a worldwide healthcare problem, its prevalence and burden have been globally increasing (1,2). Despite great advances in the management of IBD, there remain many unmet needs (3).

IBD includes Crohn's disease (CD) and ulcerative colitis (UC). UC is described by relapsing and remitting episodes of inflammation limited to the colonic mucosa, whereas CD can affect any segment of the gastrointestinal tract from the mouth to the anus (most common being the terminal ileum and colon) (1,4). CD is characterized by transmural inflammation and by skip areas of involvement. Mucosal inflammation in UC, classically begins in the rectum and spreads proximally in a continued manner (5). Although CD and UC are different, their clinical manifestations and underlying causes overlap (6).

The intestinal microbiota is a crucial component in the pathogenesis of IBD (6). Patients with IBD exhibit dysbiosis with reduced diversity of gut bacterial species (7). However, the definite causal association between IBD and dysbiosis is not yet clear to describe dysbiosis as a cause or effect (8).

Probiotics are described as ''live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'' (9). Pharmaceutical formulas of probiotic products are available over-the-counter (OTC) or by prescription. There is an increasing scientific and financial interest in the use of probiotics (10). The efficacy and safety of probiotics are being investigated in many diseases especially in gastrointestinal

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disorders. It has been reported that probiotics produce both nutritional and beneficial immune modulatory effects in IBD patients. (11).

In this study, we aimed to evaluate and compare the effectiveness of well-documented and commonly used four different probiotic microorganisms; *Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus reuteri*, *Saccharomyces boulardii* and *Bacillus clausii* in experimental colitis rat model with trinitrobenzene sulfonic acid (TNBS).

2. RESULTS

2.1. Clinical Evaluation and Change in Body Weight During Treatment

12-24 h after application of TNBS, symptoms such as diarrhea, inactivity and rectal bleeding started to appear in all groups. Loss of body weight in the colitis group was statistically significant compared to the control group (on day 4-6; $p<0.05$, $p<0.01$, and $p<0.001$, respectively). Body weight continuously and significantly increased in the control group. In the treatment groups, the change in the body weight over time was not significant ($p>0.05$) (Figure 1). No symptoms were observed in the control group. Compared to the colitis group, we observed fewer symptoms in probiotic and methylprednisolone treated groups.

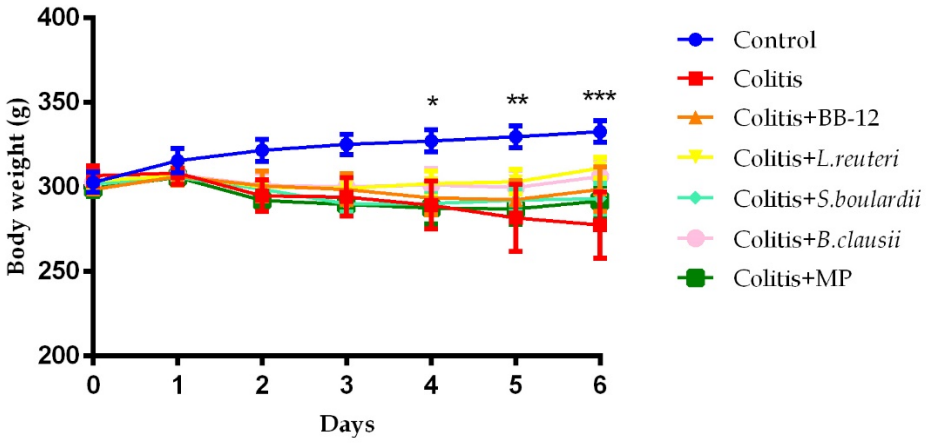

Figure 1. Body weight (g) in all animal groups during 7 days. Data were represented as mean ± the standard error of the mean (SEM). *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$ compared to the control group. MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *lactis* BB-12; *L. reuteri*: *Lactobacillus reuteri*; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*.

2.2. Disease Activity Index (DAI) Scores During Treatment

Weight loss, stool consistency, and occurrence of rectal bleeding were evaluated individually. DAI scores increased in the colitis group comparison with the control group. The maximum DAI score was observed in the colitis group. In groups of colitis+MP, colitis+BB-12, colitis+*L. reuteri*, colitis+*S. boulardii* and colitis+*B. clausii* DAI scores were low compared to the colitis group (Figure 2).

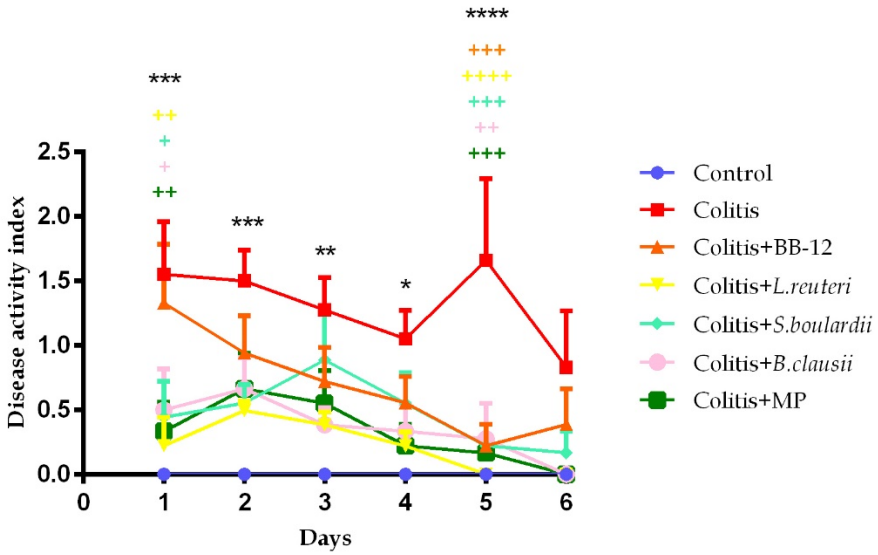

Figure 2. Disease activity index scores of all animal groups. Data were represented as mean ± the standard error of the mean (SEM). *: p<0.05, **: p<0.01, ***: p<0.001, and ****: p<0.0001 comparison with the control group; +: p<0.05, ++: p<0.01, +++: p<0.001, and ++++: p<0.0001 comparison with the colitis group. MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *lactis* BB-12; *L. reuteri*: *Lactobacillus reuteri*; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*.

2.3. Macroscopic Colonic Damage (MCD) Score

In the colitis group, swelling, edema, ulcers with inflammation, and major damage sites were observed. In treatment groups, mucosal appearance was better compared to the colitis group. MCD score was significantly increased in the colitis group comparison with the control group (p<0.0001). In groups of colitis+MP, colitis+BB-12, colitis+*L. reuteri*, colitis+*S. boulardii*, and colitis+*B. clausii*, MCD score decreased comparison with the colitis group (p<0.0001). There was no significant damage between probiotic- and methylprednisolone-treated groups (p>0.05) (Figure 3)

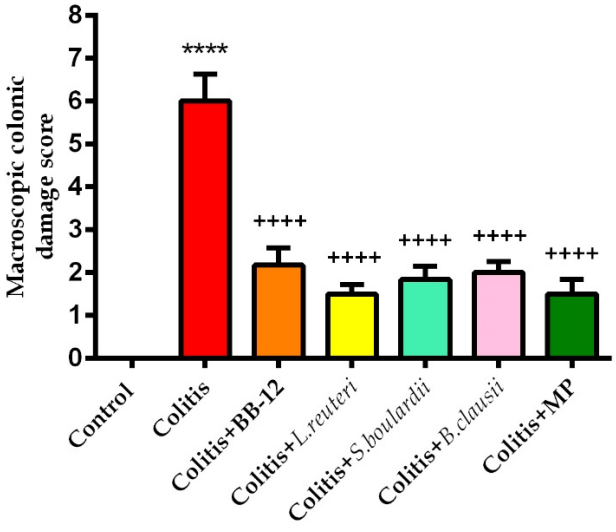

Figure 3. Macroscopic colonic damage score of groups. Data were represented as mean ± the standard error of the mean (SEM). ****: p<0.0001 comparison with the control group; ++++: p<0.0001 comparison with the colitis group. MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *lactis* BB-12; *L. reuteri*: *Lactobacillus reuteri*; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*.

2.4. Histopathological Analysis

Based on histopathological evaluations, the colonic tissues of control groups had a regular morphology (Figure 4A) with intact epithelium and glandular structures. Epithelial detachments and inflammatory cell accumulation were clearly observed in the colitis group (Figure 3B). Also, we observed submucosal granulomatosis and inflammatory cells infiltrated into the crypts in the mucosa. This process resulted in the destruction of the surface and crypt epithelium followed by atrophy and ulceration of the mucosa. Colon tissues of the treatment groups (Figure 4C-G) showed rare vascularization and mild inflammatory cells infiltrated into the submucosa. Histopathological semiquantitative score significantly increased in the colitis group comparison with the control group ($p<0.01$). The histopathological damage scores in groups of colitis+MP ($p<0.05$), colitis+ BB-12 ($p<0.05$), colitis+*L. reuteri* ($p<0.05$), colitis+*S. boulardii* ($p<0.01$) and colitis+*B. clausii* ($p<0.05$) decreased significantly comparison with the colitis group (Figure 5).

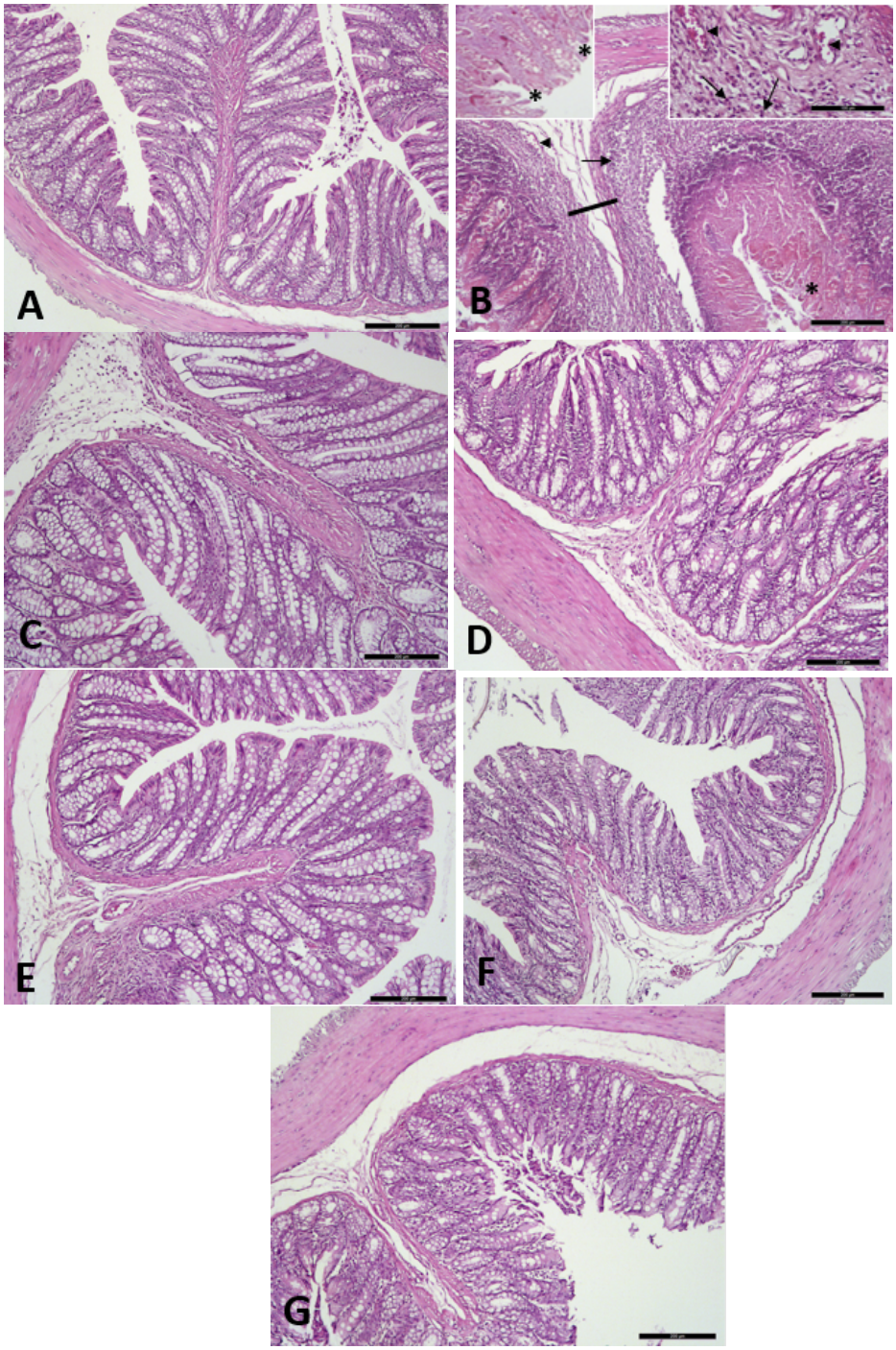

Figure 4. Hematoxylin and Eosin staining of the colon samples sections from all animal groups. A: Control group, regular colon morphology; B: Colitis group, severe inflammatory cell infiltration (arrow), degeneration of crypt structure (*), increased vascularization (arrowhead) and submucosal granulomatosis (line); C-G: Colitis+MP, Colitis+BB-12, Colitis+*L. reuteri*, Colitis+*S. boulardii* and Colitis+*B. clausii*, rare vascularization and mild inflammatory cell filtration in the submucosa. Scale bars 200 µm. MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *Lactis* BB-12; *L. reuteri*: *Lactobacillus reuteri*; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*.

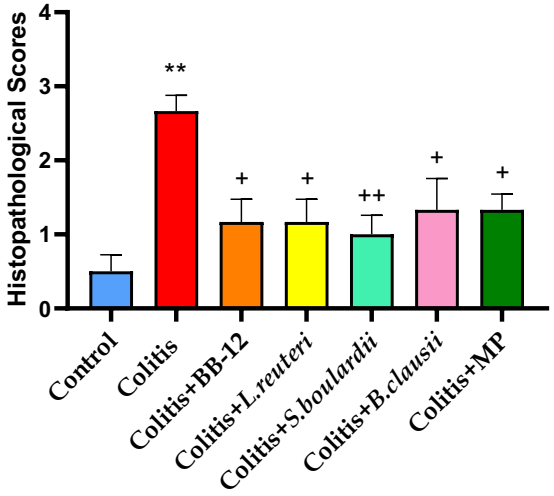

Figure 5. Histopathological semiquantitative scores of colon tissues in all animal groups. Data were represented as mean ± the standard error of the mean (SEM). **: p<0.01 comparison with the control group; +: p<0.05, and ++: p<0.01 comparison with the colitis group. MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *lactis* BB-12; *L. reuteri*: Lactobacillus reuteri; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*

2.5. MDA, GSH levels and MPO activity in the colonic tissues of groups

We evaluated the levels of malondialdehyde (MDA) and glutathione (GSH), and activity of myeloperoxidase (MPO) in the colonic tissues of groups. MDA levels increased in the colitis group comparison with the control group (p<0,001). In groups of colitis+MP (p<0,01), colitis+BB-12 (p<0,01), colitis+*L. reuteri* (p<0,01) and colitis+*S. boulardii* (p<0,05) MDA levels decreased comparison with colitis group (Figure 6).

GSH levels of the colitis group decreased compared to the control group (p<0,01). In colitis+MP (p<0,01), colitis+*L. reuteri* (p<0,05), colitis+*S. boulardii* (p<0,05), and colitis+*B. clausii* (p<0,01) groups, GSH levels increased comparison with the colitis group. In the colitis+BB-12 group, GSH levels decreased comparison with the control group (p<0,05) (Figure 6).

MPO activity of the colon tissues increased in the colitis group comparison with the control group (p<0,001). In groups of colitis+MP (p<0,01), colitis+*L. reuteri* (p<0,01) and colitis+*S. boulardii* (p<0,05), MPO activity decreased comparison with the colitis group (Figure 6).

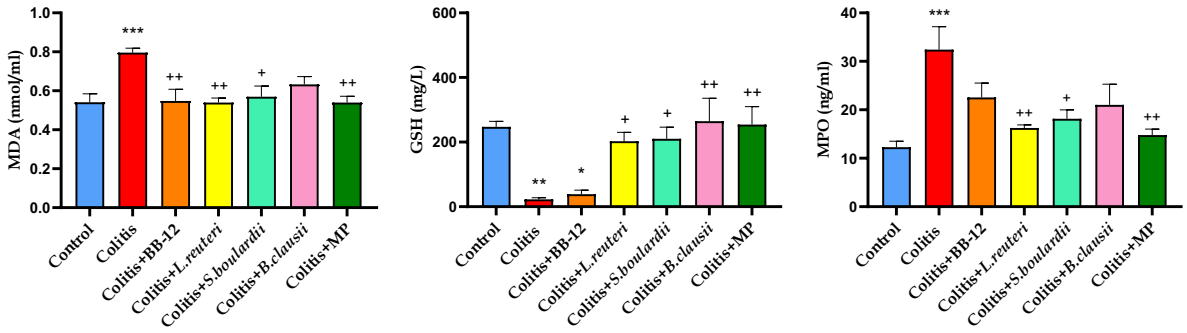

Figure 6. Levels of MDA and GSH, and activity of MPO in the colonic tissues of groups. Data were represented as mean ± the standard error of the mean (SEM). *: p<0.05, **: p<0.01, and ***: p<0.001 comparison with the control group; +: p<0.05 and ++: p<0.01 comparison with the colitis group. MDA: Malondialdehyde; GSH: Glutathione; MPO: Myeloperoxidase; MP: Methylprednisolone; BB-12: *Bifidobacterium animalis* subsp. *lactis* BB-12; *L. reuteri*: *Lactobacillus reuteri*; *S. boulardii*: *Saccharomyces boulardii*; *B. clausii*: *Bacillus clausii*.

3. DISCUSSION

IBD has turn into a global disease with growing prevalence in the 21st century (12). It poses an important social end economic burden on healthcare systems and society (13). Patients struggle with IBD's negative impact on health-related quality of life. Unfortunately, the etiopathogenesis of IBD remains unknown.

However, oxidative stress and inflammation play a crucial role in the etiopathogenesis and progression of IBD (14). The conventional treatment consists mainly of aminosalicylates, corticosteroids, immunomodulators and biologic agents (15). Unfortunately, despite these treatments, many patients do not achieve clinical remission or lose response over time (15). Probiotics' role in managing IBD is being investigated in many preclinical and clinical studies.

The use of probiotics has turn into more and more popular all over the world but one of the most important points to know about probiotics is that, not all probiotics are the same (16). There is strong evidence to support the hypothesis that the effects of probiotics are strain and disease specific (16). Furthermore, the effective dose may vary for each different indication. To solve this dilemma, there is only one option; the clinical efficacy and safety of each probiotic should be investigated and evaluated individually, like drugs. Rational use of probiotics is so crucial (right probiotic, right indication, right dose and time) (17).

Modification of the intestinal microbiota composition and activity with probiotics, the immune system, and host barrier function could be enhanced, and this is the main rationale for studies with probiotics in IBD (18). Probiotics have several mechanisms of action, such as; stimulation of the production of mucosal Ig A, competition with pathological bacteria, production of components with antibacterial activity, upregulation of tight junction molecules in the mucosal barrier, and toxin receptors' degradation (19). Specific probiotics can show antioxidant activity and diminish damages cause by oxidation (20). Probiotics influence the immune cells' activity, differentiation, antibody production and stimulate the production of anti-inflammatory cytokines and reduce pro-inflammatory cytokines (19). All these actions are dependent on the strain.

TNBS-induced colitis in rats is well documented in the literature. It applies to the group of chemically-induced colitis animal models and one of the most commonly utilized models of IBD (21,22). The model shares significant properties with IBD (especially CD, instillation of TNBS initiates ROS production which initiates and maintains colonic inflammation) and has advantages such as technical simplicity and low cost (21-23).

In our study we evaluated and compared the effectiveness of four different probiotics in TNBS induced colitis model of rats. We observed and recorded rats' general appearance, clinical symptoms, body weight, stool consistency, occurrence of rectal bleeding, and DAI score every day. Afterward we evaluated macroscopic and microscopic colonic damages and determined colonic MDA, GSH levels and MPO activity in order to examine the oxidant damage and the inflammation.

Treatment and TNBS administrations started on the same day. We observed more symptoms in the colitis group. Loss of body weight in the colitis group was statistically significant and continued gradually. In the control group there were no symptoms and body weight increased gradually and significantly. In the probiotic-treated groups, change in body weight over time was not significant. The DAI scores were low in probiotic treatment groups. Compared to the colitis group, symptoms were milder in probiotic and methylprednisolone treated groups.

In this study, histopathological and MCD scores in BB-12, *L. reuteri*, *S. boulardii* and *B. clausii* treatment groups significantly decreased compared to the colitis group. Also, the colonic damage was not significantly different between probiotic and methylprednisolone treated groups. In conclusion, severe inflammatory infiltration and degeneration of crypts resulting from colitis injury improved significantly in all treatment groups.

Oxidative damage can be measured by ELISA analysis of MDA and GSH levels. In this study, MDA and GSH measurements made in colonic tissues showed a significant decrease in MDA and a significant increase in GSH levels in the *L. reuteri* and *S. boulardii* groups compared to the colitis group. In this context, treatment with *L. reuteri* and *S. boulardii* indicate antioxidant activity. Also, treatment with BB-12 significantly decreased MDA levels and treatment with *B. clausii* significantly increased GSH levels compared to the colitis group. The activity of MPO can be used as an index of inflammation (24). In our study, colonic MPO activity significantly decreased in the *L. reuteri* and *S. boulardii* groups compared to the colitis group. Treatment with *L. reuteri* and *S. boulardii* indicate an anti-inflammatory activity.

In the literature, there are several studies that investigated probiotic effectiveness in the animal model of colitis. In one study, *Bifidobacterium infantis* showed a beneficial effect on reduction in symptoms, inflammation and mucosal damage in TNBS-induced colitis model (25). Another study showed that treatment with *Bifidobacterium bifidum* 231 revealed anti-inflammatory effects both macroscopically and histologically in TNBS-induced colitis in rats (26). A probiotic cocktail (*Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Bifidobacterium longum*) regulates the balance between anti-oxidant and oxidant systems in the colitis model (27). Based on another research; *E. feacalis*, *L. acidophilus*, *C. butyricum* and *B. adolescentis* have beneficial effects on experimental colitis in mice. Weight loss was slowed down, even weight gain was observed in the probiotic treatment groups. These probiotics decreased DAI, histological scores, and MPO activity, suggesting that they had therapeutic effects on experimental colitis (28). Our results agree with the reported findings that specific probiotics can be used in the treatment of colitis.

In clinical studies of probiotics among IBD patients, more differentiated results are noticeable (29). Some specific probiotics are recommended for the management of UC. However, for CD, there is no obvious recommendation for probiotics (15,18,30). The enthusiasm of physicians and researchers in the use of probiotics in IBD is increasing but more and well-designed studies are needed to determine which specific probiotics at which dose become more beneficial in the management of IBD (18).

4. CONCLUSION

In this study we investigated and compared four different probiotics' effectiveness in the treatment of TNBS-induced colitis in rats. According to our results, treatments with BB-12, *L. reuteri*, *S. boulardii* and *B. clausii* significantly improved macroscopic and microscopic colonic damage and clinical course. Treatment with *L. reuteri* and *S. boulardii* also significantly decreased colonic MPO activity, MDA levels and increased GSH levels. The results of this study are promising and more studies are needed to investigate the role of probiotics in the management of IBD.

5. MATERIALS AND METHODS

5.1. Probiotics and Chemicals

TNBS was purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA). Probiotics and methylprednisolone: *Bifidobacterium animalis subsp. lactis* (BB-12) drops (Linex Bakso® drop; 6 drop contains 1 x 10^9 CFU/BB-12), *Lactobacillus reuteri* drop (Biogaia® drop; 5 drop contains 1 x 10^8 CFU/*L. reuteri*), *Saccharomyces boulardii* 250 mg sachets (Reflor® sachet; lyophilized *S. boulardii*), *Bacillus clausii* spores oral suspension/vial (Enterogermina®; 5 ml contains 4 x 10^9 CFU/*B. clausii* spores) and methyprednisolone 4 mg/tablet (Prednol® 4 mg/tablet) were purchased from a local public pharmacy in Istanbul.

5.2. Animals

All animal experiments were performed with the approval of the Marmara University Animal Experiments Local Ethics Committee with permission number: 20.2021.mar. Forty-two male Wistar albino rats (300-340 g) (n=6 in each group) were picked up from Kobay Experimental Animals Lab., Ankara, Turkey. The rats were lived under controlled temperature (20±2 °C), in humidity (40-60 %) and light (12 h/12 h light/dark regime)-regulated rooms. The animals were kept on a standard rodent pellet diet, with tap water available ad libitum.

5.3. Experimental Design of Study

Rats were randomly divided into seven groups, with six rats per group as follows:

A. Control group: Healthy control group, received 25% aqueous ethanol once at a dose of 1 ml/kg into the colon on day 0 and intragastric distilled water at a dose of 2 ml/kg/day for 7 days.
B. Colitis group: Untreated TNBS-induced colitis control group received TNBS (80 mg/kg) (i.c) on day 0, and received i.g. distilled water at a dose of 2 ml/kg/day for 7 days.
C. Colitis + BB-12: Animals received TNBS (80 mg/kg) (i.c) on day 0 and 1 x 10^9 CFU/day *Bifidobacterium animalis* subsp. *lactis* BB-12 (i.g) for 7 days.
D. Colitis +*L. reuteri*: Animals received TNBS (80 mg/kg) (i.c) on day 0 and 1 x 10^8 CFU/ day *L. reuteri* (i.g) for 7 days.
E. Colitis + *S. boulardii*: Animals received TNBS (80 mg/kg) (i.c) on day 0 and *Saccharomyces boulardii* 10 mg/kg/day (i.g) for 7 days.
F. Colitis + *B. clausii*: Animals received TNBS (80 mg/kg) (i.c) on day 0 and 1 x 10^9 CFU/day *Bacillus clausii* spores (i.g) for 7 days.
G. Colitis + MP: TNBS-induced colitis treated with methylprednisolone received TNBS (80 mg/kg) (i.c) on day 0 and intragastrically methylprednisolone at a dose of 1 mg/kg/day for 7 days.

All treatments and TNBS administration began on day 0 of the experiment. Probiotic and methylprednisolone treatments continued daily for 7 days by intragastric gavage.

5.4. Induction of Colitis: Intracolonic Injection of TNBS

Rats were starved for 24 h but had free access to water before induction of colitis. Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.; Pfizer) and xylazine hydrochloride (10 mg/kg, i.p.; Bayer). A flexible polypropylene catheter was inserted rectally into the colon with the tip approximately 8 cm proximal to the anus. For the induction of colitis 80 mg/kg (8 mg/100 g/0.2 ml dissolved in %50 ethanol, solution 0.2 ml/100 g in volume) TNBS was administered via catheter (31-32). The rats were kept in trendelenburg position for 5 minutes to prevent anal leakage.

5.5. Disease Activity Index

DAI scoring was calculated in accordance with body weight loss (as a percentage), differences in stool consistency, and the existence of rectal bleeding, as described previously (Table 2) (31).

Table 2. Scoring of disease activity index (DAI)

Score	Weight loss (%)	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1-5		
2	5-10	Loose stools	
3	10-20		
4	>20	Diarrhea	Gross bleeding

DAI value is the combined scores of weight loss, stool consistency and bleeding divided by 3

5.6. Sacrification of Animals and Removal of Colon Tissue

At the end of the experiment, all rats were decapitated 1 week after induction of colitis and the colon was removed for the assessment of colonic damage, scored for macroscopically visible damage, and histopathological and biochemical analysis.

5.7. Macroscopic Colonic Damage Score

The colon was removed for the assessment of colonic damage and scored for macroscopically visible damage as described previously (Table 3) (31).

Table 3. Criteria for scoring 'macroscopic mucosal damage' component of combined damage score

Score	Appearance
0	Normal
1	Localized hyperemia, no ulcers
2	Ulceration without hyperemia or bowel wall thickening
3	Ulceration with inflammation at one site
4	Two or more sites of ulceration and inflammation
5	Major sites of damage extending >1 cm along length of colon
6-10	Major sites of damage extending >2 cm along length of colon, with score increasing by 1 for each additional cm

5.8. Light Microscopy Preparation and Histopathological Scoring

For the histological examination, colon samples were fixed in 10% neutral-buffered formalin for at least 48 h. Then, tissue samples were dehydrated in graded ethanol series (%70, 90, 96 and 100), cleared in xylene, embedded in paraffin and sliced into 5 µm sections. Stained sections from each rat and five similar areas in each section were analyzed by experienced histologists blinded to the experimental groups. Followed by staining with hematoxylin and eosin (H&E) for light microscopic examination to assess colon injury. The sections were then examined and photographed with a light microscope (Leica DM 1000). H&E-stained all tissue sections were scored semiquantitatively using a scale ranging from 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe). Modified histopathological scoring criteria included, degeneration of surface and crypt epithelium, degeneration of villus structure, and inflammatory cell infiltration for the colon (33).

5.9. Biochemical analysis

Tissue samples were collected after sacrification and biochemically analyzed using ELISA kits (Elabscience and Mybiosource). All procedures were carried out according to the manufacturer's instructions. Levels of MDA and GSH, and activity of MPO were measured in colon tissues.

5.10. Statistical analysis

Data analysis was done using GraphPad Prism 9.5.0 software (San Diego, USA). The results of the tests were analyzed using ANOVA to compare the mean of measurements over time, one-way ANOVA or two-way ANOVA to compare the mean of measurements between groups and Tukey's method for pairwise comparison in case of difference. Data were represented as mean ± the standard error of the mean (SEM). $p<0.05$ values were respected significant.

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Author contributions: Concept – E.K., A.N.H.Y., L.K.; Design – E.K., A.N.H.Y., L.K.; Supervision – E.K., A.N.H.Y., L.K.; Resources – E.K.; Materials – E.K., A.N.H.Y.; Data Collection and/or Processing – E.K., A.N.H.Y., G.S.K., Y.Y; Analysis and/or Interpretation – E.K., A.N.H.Y., G.S.K., Y.Y., L.K; Literature Search – E.K.; Writing – E.K., G.S.K.; Critical Reviews- E.K., A.N.H.Y., G.S.K., Y.Y., L.K.

Conflict of interest statement: The authors declared no conflict of interest.

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