

The beneficial role of the probiotic *Bacillus clausii* on induced Murine Colitis: a clinical and pathological study

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Abstract

This study was performed to investigate inflammatory bowel disease (IBD), especially ulcerative colitis in a murine model by using TNBS-induced colitis; therefore it was performed on 60 animals divided equally into five groups, the first group was the control which received standard food and water only, the second group was probiotic which used 100 µl of *Bacillus clausii*, the third group disease group by TNBS induced colitis, the fourth group probiotic with TNBS induce colitis orally, the fifth group probiotic with TNBS induce colitis intra-rectal; the results showed loss of body weight and increased in body temperature in disease group TNBS compared to control groups during the duration of the research, the macroscopical features showed the shortness of colon in length, as well as swollen and congestion of colon in the disease induced group compared to other control and probiotic groups; the micropathological results of colon showed sloughing of mucosal layer, sever hyperplastic goblet cells of the mucosal layer of the colon by PAS stain in the disease induced group compared to other control and probiotic groups. the current study concluded that whether *Bacillus clausii* can prevent TNBS-induced colitis which is associated with microbial dysbiosis and sought to understand its origins; as a consequence, probiotic treatment reduced colonic inflammation and By increasing a group of gram-positive bacteria, researchers reduced the microbial dysbiosis that TNBS Induce-colitis causes.

Keywords: colitis, probiotic, TNBS, *Bacillus clausii*.

Introduction

Chronic inflammation illness with an unknown cause, inflammatory bowel disease. There are two primary clinical forms of IBD that are recognized: Crohn's disease (CD) and ulcerative colitis (UC) (Eichele and Young.,2019). While CD affects any portion of the gastrointestinal system intermittently, UC restricts inflammation to the colon and rectum (Ray and Sagar.,2020). Both kinds are chronic, inflammatory diseases that grow over time and frequently have remissions and relapses (Flynn and Eisenstein.,2019). Uncertainty surrounds the pathophysiology of UC and CD, however intricate connections between genetic, environmental, immunological, and gut microbial variables have been proposed. They coordinate a series of inflammatory reactions in the gut mucosa through their interactions (Kuhnen.,2019). It has been proven that immune cells release active substances linked to the start and maintenance of inflammation and the destruction of gastrointestinal tissue. Additionally, it has been discovered that IBD patients' colonic mucosa has an excessive infiltration of immune cells (Zhang and Li.,2014).

The etiology of UC and CD has also been linked to aberrant regulation of numerous cytokines. The cytokines interleukin (IL)-17A and IL-23 are among

them (Lopez et al.,2015). Mucosal edema and hemorrhagic ulcerations were found during the colon and rectum were examined under a microscope up to 24 hours following the TNBS therapy. The mucosa and submucosa's histologic examination in the first week revealed a significant infiltration of inflammatory cells. Neutrophils, and lymphocytes penetrated the mucosa, submucosa, and muscle layers (Buonocore et al.,2010). The probiotic bacteria *Bacillus clausii* is spore-forming, rod-shaped, aerobic, Gram positive, acid-resistant, and capable of colonizing the gut (Ghelardi et al.,2015). A combination of four *Bacillus clausii* probiotic strains (O/C, T, SIN, and N/R) may be useful in treating inflammatory bowel disease, according to data (Ianiro et al .,2018).

Material and methods

The female mice used in this study were aged between 8 and 10 weeks were used for study, all mice were housed at the University of Basrah, veterinary medicine animal facilities, residing in a designated pathogen-free environment with 12-hour light/dark cycles, all mice were housed in rooms with controlled temperature, ventilation.

A total of 60 female mice (Balb\c) which treated for 7 days as the following: To induce colitis 1 mg of (TNBS) injected into 90% ethanol will be

administered intrarectally to mice in a 50µL volume total . (Bang&Lichtenberger.,2016). For treatment groups, mice will be given 100 µL oral gavages of *Bacillus clausii* (O/C, SIN, N/R, T) probiotics. parameters of the colitis disease assessment. Mice were weighed every day for both colitis models, and colon lengths were measured at the conclusion of the experiment. The experimental groups' macroscopic colitis scores were determined using a slightly modified scoring system. IL-22 neutralization or induction of colitis and therapy with *Bacillus clausii* (O/C, SIN, N/R, T). In order to induce TNBS colitis in groups, mice were either administered 100 µL of probiotics intrarectally or orally, 24 hours after injection of 1 mg of TNBS in 90% ethanol (Elson et al.,1995). Every day for the duration of the experiment, 100 µL of subsequent treatments were administered *Bacillus clausii* (O/C, SIN, N/R, T) dose and administration route are similar with our earlier studies (Rouse et al., 2013). 50% ethanol intrarectal injections were provided to all experimental groups using the TNBS model to make sure any changes weren't caused by BALB/c mice. 100 µL of TNBS (Sigma-Aldrich) dissolved in 90% ethanol were administered intrarectally to mice in total for treatment as a result of the ethanol diluent administration.Colon tissues are examined under a microscope. The TNBS model reached day four, the proximal colon was harvested to get the colon histology. The tissues were embedded in paraffin after being fixed in a 10% formalin After 24 hours, then remove the fixative solution and cut into 9-m pieces (Suvarna et al.,2018). Sections were -+stained as previously mentioned in preparation for H&E staining. Histopathological criteria were scored based on previously published findings. Sigma-Aldrich provided a staining kit for usage in accordance with the manufacturer's instructions for periodic acid-Schiff (PAS) staining. A 38 mm catheter was used to inject 1 mg of TNBS, which was diluted in 0.1 mL of 50% ethanol, into a moderately anesthetized (5% isoflurane) for overdose inhalation . The TNBS was acquired from Sigma-Aldrich (MO, USA).

Results

Clinical Parameter

The current study examined the effectiveness of *Bacillus clausii* to prevent a chemically induced murine colitis model with TNBS and examine at the potential probiotics prevent colitis.Following the TNBS-induced disease, *Bacillus clausii* was administered orally to the treated groups daily after 24 hours.According to the results of the study, mice with colitis (TNBS) significantly lost weight (17% less than control groups Naive or Pro), whereas animals with colitis given *Bacillus clausii* experienced the opposite effect (TNBS+Pro O or TNBS+ Pro A) had significantly decreased incidence of weight loss ($P<0.05$, $P<0.01$) (Figure 1A).

A physical stool examination was also performed

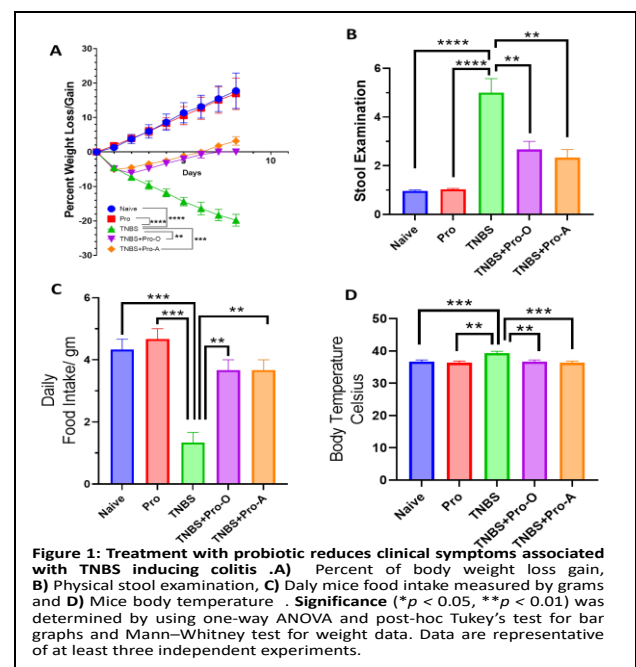
including color, consistency, and blood contained in stool, colitis mice (TNBS) showed a significantly increased in stool scoring level compared to controls groups (Naïve or Pro) ($P<0.001$) and colitis treatments groups (TNBS+Pro O or TNBS+Pro A) ($P<0.01$). (Figure 1B). Daily mice food intake was measured in grams.

The data showed colitis mice (TNBS) showed significantly decreasing levels of food intake ($P<0.001$) where food intake was less than 2 grams compared to controls groups (Naïve or Pro) where food intake was 5 to 6 grams daily and colitis treatments groups (TNBS+Pro O or TNBS+Pro A) were food intakes was 3.5 to 4 grams daily ($P<0.01$) (Figure 1C).

Mice body temperature is one of the very important sings that associated with inflammation and the experimental data showed there were a significantly increased in mice body temperature in the disease group (TNBS) 39 Celsius conversely to controls groups (Naïve or Pro) and colitis treatment groups (TNBS+Pro O or TNBS+Pro A) which were near to normal 36-37 Celsius ($P<0.01$, $P<0.001$) (Figure 1D).

Macroscopically results of visceral organs:

The macroscopic effects of colitis are lessened with *Bacillus clausii* therapy. In the current investigation, we used TNBS to examine how well *Bacillus clausii* prevented a chemically induced mouse colitis model. Treatment groups received oral doses of *Bacillus clausii* 24 hours after disease induction by TNBS in order to study the prevention of disease by probiotic substance. The mesenteric lymph nodes in colitis mice (TNBS) significantly enlarged and congested during the experimental of the research in comparison to control groups (Naive or Pro) showed figure (2A), conversely of colitis mice administrated with *Bacillus clausii* (TNBS+Pro O or TNBS+ Pro A) had significantly decreased incidence of irregular arrangement of visceral organ and mesenteric lymph node become natural state showed figure (2B).



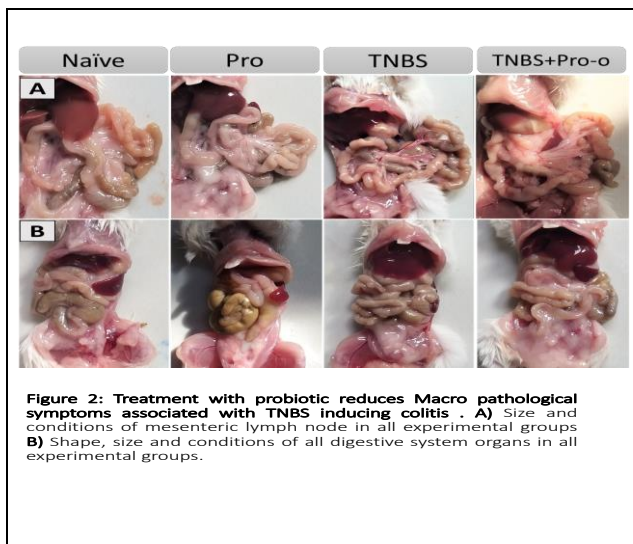


Figure 2: Treatment with probiotic reduces Macro pathological symptoms associated with TNBS inducing colitis . A) Size and conditions of mesenteric lymph node in all experimental groups B) Shape, size and conditions of all digestive system organs in all experimental groups.

Macroscopically results of the colon

The macroscopic effects of colitis are lessened by treatment with *Bacillus clausii* With the help of TNBS .In the current study, we examined the effectiveness of *Bacillus clausii* in preventing a murine colitis model that was chemically produced. After disease induction by TNBS, treatment groups received daily oral doses of the medication after receiving *Bacillus clausii* after 24 hours, in order to study the prevention of disease by probiotic substance. In contrast to the control groups (Naive or Pro), colitis mice (TNBS) had significantly shorter colons that were swollen and congested throughout the course of the experiment, shown in figure (3A), conversely of colitis mice administrated with *Bacillus clausii* (TNBS+Pro O or TNBS+ Pro A) had significantly decreased incidence of colon length, size ($P < 0.05$, $P < 0.01$) become natural state showed figure(3B).

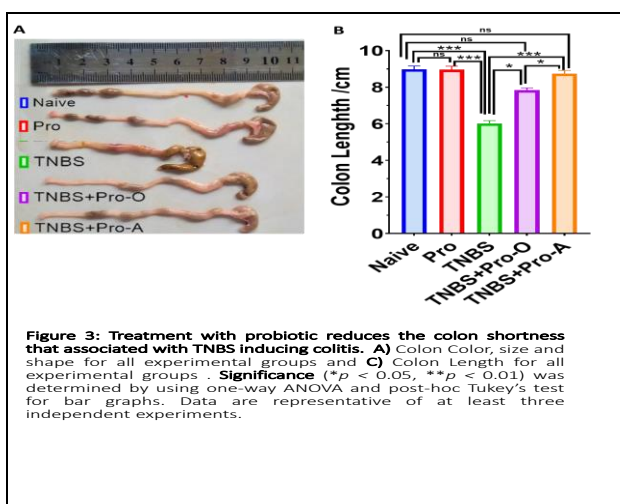


Figure 3: Treatment with probiotic reduces the colon shortness that associated with TNBS inducing colitis. A) Colon Color, size and shape for all experimental groups and C) Colon Length for all experimental groups . Significance ($*p < 0.05$, $p < 0.01$) was determined by using one-way ANOVA and post-hoc Tukey's test for bar graphs. Data are representative of at least three independent experiments.**

Microscopical results

The microscopical results of the colon of the current study showed a normal appearance of the colon layer which consists of the normal mucosal layer, normal sub mucosal layer, normal muscular layer, and normal serosa layer in the control group (G1) as in figure (4A). The histopathological transverse section (Swiss roll technique) of the colon of the control group (G1) showed a normal appearance of

the colon layer which consists of a normal mucosal layer, normal submucosal layer, normal muscular layer, and normal serosa layer as in figure (4B). In other section of the control group (G1) showed a normal appearance of the colon layer which consists of a normal mucosal layer and a normal submucosal layer by using periodic acid shift as in figure (4C).

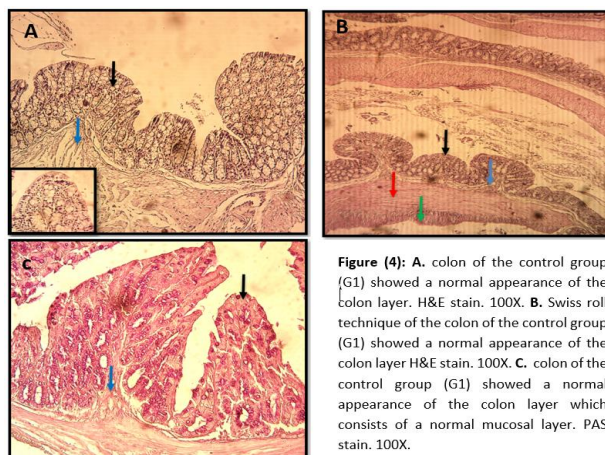


Figure (4): A. colon of the control group (G1) showed a normal appearance of the colon layer. H&E stain. 100X. B. Swiss roll technique of the colon of the control group (G1) showed a normal appearance of the colon layer H&E stain. 100X. C. colon of the control group (G1) showed a normal appearance of the colon layer which consists of a normal mucosal layer. PAS stain. 100X.

The microscopical results of the colon of the probiotic group (G2) showed a normal appearance of the colon layer which consists of the normal mucosal layer with mild inflammatory cells infiltration and normal muscular layer, normal serosa, and normal submucosal layer with mild inflammatory cells infiltration as in figure (5A), in other section of the colon of the probiotic group (G2) showed normal appearance of colon layer which consist of the normal mucosal layer with inflammatory cells infiltration in the intestinal lumen, normal submucosal layer, normal muscular layer and normal serosa layer as in figure(5B). The histopathological of the colon of the probiotic group (G2) showed a normal appearance of the colon layer which consists of a normal mucosal layer, normal submucosal layer, normal muscular layer, and normal serosa by PAS stain as in figure(5C).

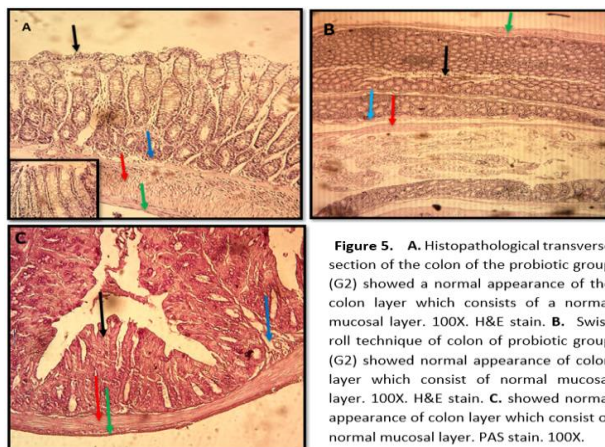


Figure 5. A. Histopathological transverse section of the colon of the probiotic group (G2) showed a normal appearance of the colon layer which consists of a normal mucosal layer. 100X. H&E stain. B. Swiss roll technique of colon of probiotic group (G2) showed normal appearance of colon layer which consist of normal mucosal layer. 100X. H&E stain. C. showed normal appearance of colon layer which consist of normal mucosal layer. PAS stain. 100X.

The histopathological of the colon of TNBS diseased group (G3) showed de-epithelization and with sloughing of the mucosal epithelial layer, severe infiltration of inflammatory cells, mainly polymorphonuclear cells, in the mucosal and submucosal layer as in figure(6A). in other sections of the colon of TNBS diseased group (G3) showed

severe de-epithelization and sloughing mucosal epithelial layer, as well as a significant infiltration of inflammatory cells as in figure(6B). The Histochemical of the colon of TNBS diseased group (G3) showed a

positive PAS stain in the severe diffused hyperplastic goblet cells of the mucosal layer of the colon by PAS stain as in figure (6C).

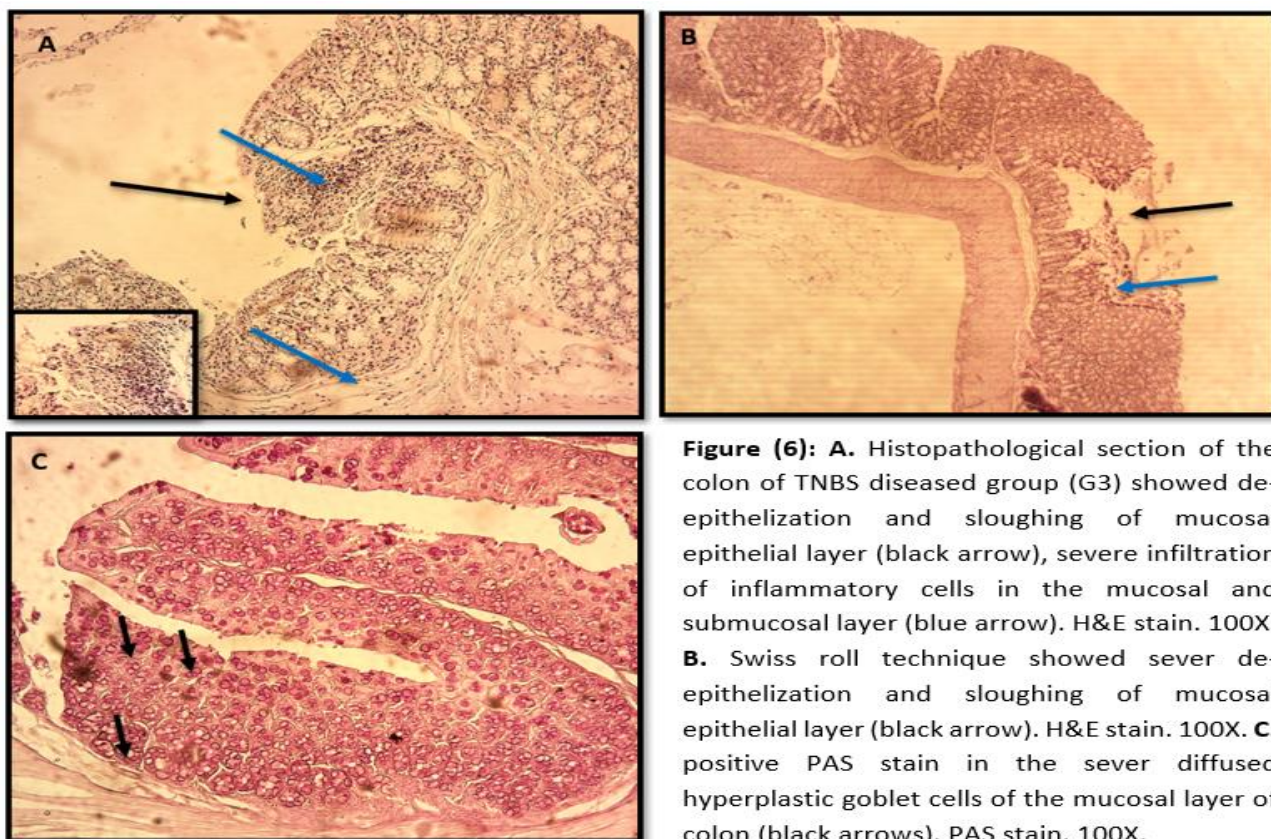


Figure (6): A. Histopathological section of the colon of TNBS diseased group (G3) showed de-epithelization and sloughing of mucosal epithelial layer (black arrow), severe infiltration of inflammatory cells in the mucosal and submucosal layer (blue arrow). H&E stain. 100X. B. Swiss roll technique showed severe de-epithelization and sloughing of mucosal epithelial layer (black arrow). H&E stain. 100X. C. positive PAS stain in the severe diffused hyperplastic goblet cells of the mucosal layer of colon (black arrows). PAS stain. 100X.

The histopathological of colon of oral TNBS + probiotic group (G4) showed re-epithelization of mucosal layer of colon with infiltration of inflammatory cells and mild edematous fluid in the submucosal layer as in figure (7A). In other section of colon of oral TNBS + probiotic group (G4) showed re-epithelization of mucosal layer of colon with infiltration of inflammatory cells and mild edematous fluid in the submucosal layer as in figure (7B). The histochemical of colon of oral TNBS + probiotic group (G4) showed positive PAS stain in the mild hyperplastic goblets cells of mucosal layer of colon as in figure(7C).

consist of normal goblet cells in the mucosal layer as in figure(8A). In the other section of the colon of anal TNBS + probiotic group(G5) showed re-epithelization of colon mucosal layer, normal architecture of submucosal and muscular layer as in figure(8B). The Histochemical of colon of anal TNBS + probiotic group (G5) showed normal appearance of colon layer which consist of normal goblet cells in the mucosal layer by using PAS as in figure (8C).

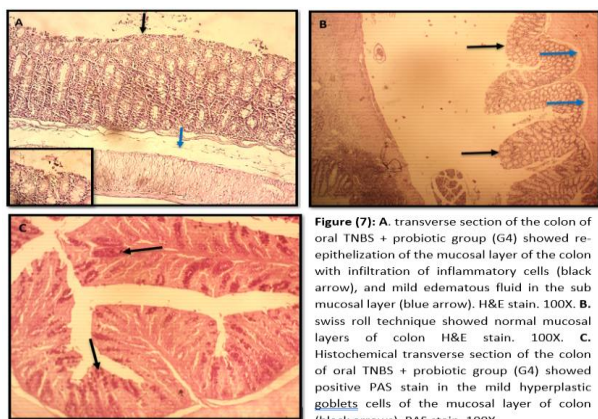


Figure (7): A. transverse section of the colon of oral TNBS + probiotic group (G4) showed re-epithelization of the mucosal layer of the colon with infiltration of inflammatory cells (black arrow), and mild edematous fluid in the submucosal layer (blue arrow). H&E stain. 100X. B. swiss roll technique showed normal mucosal layers of colon H&E stain. 100X. C. Histochemical transverse section of the colon of oral TNBS + probiotic group (G4) showed positive PAS stain in the mild hyperplastic goblets cells of the mucosal layer of colon (black arrows). PAS stain. 100X.

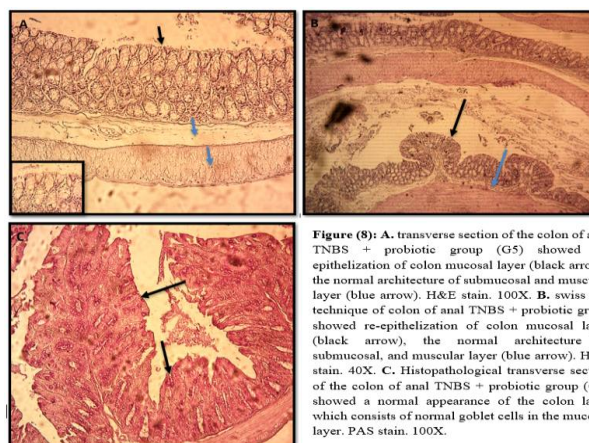


Figure (8): A. transverse section of the colon of anal TNBS + probiotic group (G5) showed re-epithelization of colon mucosal layer (black arrow), the normal architecture of submucosal and muscular layer (blue arrow). H&E stain. 100X. B. swiss roll technique of colon of anal TNBS + probiotic group showed re-epithelization of colon mucosal layer (black arrow), the normal architecture of submucosal, and muscular layer (blue arrow). H&E stain. 40X. C. Histochemical transverse section of the colon of anal TNBS + probiotic group (G5) showed a normal appearance of the colon layer which consists of normal goblet cells in the mucosal layer. PAS stain. 100X.

The colon of anal TNBS + probiotic group (G5) showed normal appearance of colon layer which

Discussion

Clinical observation

In the current study, many important clinical signs were noted in animals with TNBS induced colitis, in

general, represented by loss of body weight, increase temperature, and diarrhea with blood which due to inflammation in the colon decrease absorption that may decrease body weight compared to control groups, these results agreed with Al rafas *et al.*, (2020) who mentioned that the investigated the efficiency of resveratrol to treat a murine colitis model that was chemically induced using TNBS, as well as the investigation of the disease prevention using this natural substance via resveratrol 24 hours prior to disease induction by TNBS, followed by daily oral administrations of the treatment; as shown, murine colitis had significant weight loss (15%) during the study compared to the control group, but murine colitis pretreated with resveratrol had significantly decreased incidence. In addition, TNBS mice had much shorter colons than controls, which is thought to be another characteristic of many colitis models.

The prevention of colitis by using probiotic of *Bacillus clausii* in treated groups after TNBS causes the disease, and then the treatment is administered orally every day. and intra rectal which decrease in body weight significantly agreed with Nista *et al.*, (2021) and Ciprandi, (2004) who mention the use of *Bacillus clausii* as probiotic application on level of gut microbiota in dysbiosis which effect inducing the Th1 response in the patient's mucosa, which has a positive anti-inflammatory and immunomodulatory impact on mucosal cytokines and reduces pathogenic bacteria, helps in reducing symptoms of dysbiosis associated with it, such as diarrhea and intestinal discomfort due to antibiotic therapy, this observed after inducing colitis by DDS and treatment with probiotic.

The five groups of mice: Disease group (TNBS induce colitis), disease with treatment (oral administration) group, a naïve group (control group), a probiotic group with *Bacillus clausii*, and a disease with treatment (intrarectal) that observed reduce in body weight compared with treatment group and notice TNBS induce colitis also significantly colon's length shortening in comparison to control groups nevertheless, probiotic treatment for colitis, *Bacillus clausii* prevented this colon-shortening effect this occurs due to inflammation of the colon Plevy *et al.*, (2013) and Hameed *et al.*, (2022) who mention that inflammation of IBD effect on colon shortness.

The signs of TNBS induce colitis noticed diarrhea and weight loss and abdominal pain due to inflammation of the colon which agreement with (Jergens *et al.*, 1992; Washabau *et al.*, 2010) that showed the phrase "canine idiopathic inflammatory bowel disease" refers to a number of disorders marked by recurring or persistent gastrointestinal symptoms. diarrhea, weight loss with no underlying cause and histological evidence of mucosal inflammatory infiltration.

Histopathology observation

The present study disclosed that certain histological features including crypt atrophy are significant in the

diagnosis of TNBS induce colitis due to inflammation these results agreed with Surawicz *et al.*, (1994) who noticed that forming of crypts, atrophy of the colon and the mucosa of the bowel is wet from blood and mucus with hemorrhage, ulcers of different size and shapes due to inflammation, this investigation which agreed with Petras, (2015) and Hameed, (2022) who mention that mucosa layer contains blood, mucus, and petechial hemorrhage and ulcer.

The inflammatory cells infiltrated and increased in lamina propria due to inflammation of the colon and necrotizing effects of the TNBS which caused crypt abscess by the accumulation of inflammatory cells at the base of crypts that agree with Goldblum, (2018) and Ahmed *et al.*, (2022) who mentioned the infiltration of inflammatory cells increase in lamina propria and crypt abscess by the accumulation of inflammatory cells which lead to decrease in mucus and irregular shape crypts.

Microscopically study

The tissues were damaged in the colon as well as the inflammation and inflammatory cells infiltration, tissue lesions were evaluated in the colon due to TNBS induce colitis in the diseased group while significantly decrease lesions in treatment groups with probiotic of *Bacillus clausii* compared to the control group this results agreed with Benson *et al.*, (2012) and Saeed *et al.*, (2022) who mentioned that inflammation of colon and infiltration of inflammatory cells increase in the colon due to TNBS induce colitis and when treatment with probiotic lead to decrease this lesion.

The microscopic examination showed the colitis mice treated with probiotics had less severe scores and observations than the controls or the TNBS group, which experienced tissue sloughing and ulcerations in the colon lining. When compared to control, histological investigation of the diseased mice revealed tissue degradation in the mucosa and lamina propria layers, loss of crypts, and more cellular infiltration than in the healthy animals. These findings are consistent with Busbee *et al.*, (2019); Hameed and Ahmed (2021) who observed the microscopic investigation showed that the TNBS group displayed signs of ulcerations and tissue sloughing in the lining of the colon, but same observations were much less noticeable in colonic linings of control or colitis mice treated with probiotics. The present histological analysis supported these microscopic findings by showing that sick animals had overall tissue degradation in the mucosa, submucosa, and lamina propria layers, loss of crypts, and greater signs of cellular infiltration as compared to controls. However, the colons of mice with TNBS-induced colitis that received probiotic treatment maintained their crypt development and normal colonic tissue architecture, and they had less cellular infiltration.

The goblet cells were reduced in TNBS-induced colitis due to inflammation of the colon which causes these defects it showed the goblet cells increased in

number at probiotic uses, therefore the treated group showed normal function regarding the excretion of mucins such as MUC2 these results agreed with Kim & Ho., (2010) and Knoop et al., (2015) who found that goblet cells & enteroendocrine cells are secretive cells after using of probiotics.

Conclusions

The current study concluded the murine colitis model induced by TNBS can ameliorated by using probiotics like *Bacillus clausii*. The body weight and other related symptoms&signs were significantly neutralized to the level of control after using of the probiotics *Bacillus clausii*. The main pathological lesions associated with colitis were dropped by the ameliorating effects of probiotics.

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