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Biochemical and histological evaluation of a novel *Lactiplantibacillus plantarum* strain as a promising probiotic therapy for treating IBD induced by acetic acids in rats

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Abstract

Inflammatory bowel disease (IBD) is a chronic gastrointestinal condition with significant impacts on quality of life. Current treatments, including anti-inflammatory drugs and immunosuppressants, often cause severe side effects, emphasizing the need for safer alternatives. Probiotics offer a promising therapeutic option due to their ability to modulate gut microbiota and reduce inflammation. This study evaluates the prophylactic and therapeutic potential of a novel *Lactiplantibacillus plantarum* C4 strain, isolated from milk, in an acetic acid-induced IBD rat model. Rats were divided into five groups, five animals each. Animal groups were negative control, positive control (induction only), and three treatment groups receiving sulfasalazine, a commercial probiotic, or the novel *Lactiplantibacillus plantarum* C4 strain. Efficacy was assessed through histological examination and biochemical analysis, including inflammatory biomarkers (IL-6, IL-1β, TNF-α) and oxidative stress markers (superoxide dismutase, catalase, and malondialdehyde). The novel *Lactiplantibacillus plantarum* strain demonstrated comparable effectiveness to sulfasalazine in mitigating histological damage, reducing inflammatory marker levels, and restoring antioxidant enzymes activity. These findings suggest that the novel *Lactiplantibacillus plantarum* C4 holds significant promise as a safe, effective and accessible therapeutic option for IBD.

Keywords Inflammatory bowel disease, Inflammation, Oxidative stress, Histopathology, Probiotics

1 Introduction

Inflammatory bowel disease (IBD) is a chronic condition that affects the GIT causing inflammation episodes. This inflammation is mostly due to abnormal immune response to the GIT microflora [1, 2]. IBD affects around 7 million people around the world with an increase of around 90% in the last 30 years [3]. IBD is usually sub classified to Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease can affect any part of the GIT but mostly the small intestine and the upper part of the colon while UC causes the

inflammation in large intestine. Both CD and UC cause uncomfortable symptoms like abdominal pain, diarrhea or constipation, bloating, upset stomach, nausea and vomiting, unexplained weight loss and UC may cause bloody stool as well [4]. Besides these symptoms, which cause a serious and long term decrease of the quality of life, IBD patients also has a higher risk to develop serious complications if they don't receive effective treatment, the complications include intestinal abscess, anal fistula, strictures, perforated bowl, toxic megacolon and even colon cancer [5].

Human gut microbiota was found to be closely related to intestinal disease occurrence, development, and prognosis [6]. Many studies have discussed the role of gut microbiota dysbiosis in the pathogenesis of IBD and other GIT diseases. Dysbiosis being a consistent feature in patients suffering from Crohn's disease (CD) and ulcerative colitis (UC) [7]. This dysbiosis involves the change in several bacteria such as the decrease of *Faecalibacterium prausnitzii* [8] and *Firmicutes* and the increase in *Escherichia coli (E. coli)* and *Proteobacteria* [7, 9]. The imbalance in gut microbiota might contribute in the pathogenesis of IBD in several ways such as disruption of intestinal barrier [7], induction of cytokines release by immune cells [8] and even alteration of some metabolites level, including the decrease in the production of short-chain fatty acid by bacterial fermentation of undegistable carbohydrates. The decrease of such beneficial metabolites also influences immune regulation and epithelial barrier integrity [10]. The effect of dysbiosis may be also enhanced by other host genetic and environmental factors. Driven by this information gut microbiota modulation may represent a promising solution for IBD with minimum or no side effects. Therapeutic strategies targeting the microbiota, such as probiotics, prebiotics, and fecal microbiota transplantation, are being explored for their potential to restore microbial balance and reduce disease activity [11, 12].

Probiotics are proven to have positive effect on IBD by the modulation of inflammation, oxidative stress [13] and host immune interaction. In addition, probiotics are very easy to be administrated as food supplement or in dairy products with no or very mild adverse effect. These features represent probiotics as a superior alternative for conventional treatments of IBD which are usually combined with serious side effects. For example, 5-aminosalicylates may cause side effects like diarrhea, nausea and abdominal pain and more serious complications such as oligospermia and pancreatitis [5, 14], corticosteroids could cause osteoporosis and hypertension with long term use and immunosuppressant increases the risk of infection and even malignancy [15]. Even biologics like infliximab requires to be administered intravenously and may cause serious allergic reactions [16].

Previous studies discussed the safety and efficacy of probiotics in the treatment of IBD [17], tested species belongs to different genera *Lactobacillus*, *Bifidobacterium*, *and Saccharomyces. Lactobacillus* showed significant improvement in the level of inflammatory biomarkers in the treated subjects [18].

In this study, a novel *Lactiplantibacillus plantarum* C4 strain isolated from dairy products and showed prominent protective effect against different pathogenic strains of bacteria [19] was tested for its prophylactic effect against bowel inflammation induced by acetic acid. The effect was evaluated biochemically and histopathologically to estimate the opportunity of the novel studied *Lactiplantibacillus plantarum* strain to be effectively used as a prophylactic and therapeutic agent against IBD.

2 Experimental

2.1 Materials

MRS broth culture media for growth and cultivation of *Lactobacillus*, acetic acid for induction of bowel inflammation, and sulfasalazine, for the treatment were purchased from Sigma-Aldrich. Enterogermina oral vial, containing spores of *Bacillus Clausii* (2 billion units/5 ml), was purchased from local market for comparison with our novel *Lactiplantibacillus plantarum* strain. ELISA kits for determination of rat IL-1β, IL-6 and TNF-α were purchased from Elabscience. Lipid peroxidation, malondialdehyde (MAD), kit was purchased from sigma-Aldrich. Our novel probiotic strain, *Lactiplantibacillus plantarum* C4 isolate [19] was cultured in MRS broth for 24 h in an anaerobic condition and bacterial cells were obtained by centrifugation of the bacterial cultures at 1000 rpm for 5 min, washed twice with saline and then suspended in PBS to obtain (2 billion CFU/5 ml) suspension.

2.2 Animal models and experimental design

The study was conducted on adult male Wistar albino rats weighing 140–150 g, obtained from VACSERA, Egypt, and housed under controlled, pathogen free conditions at animal facility of MSA University, Egypt. The animals were maintained at a constant temperature of 24 °C with a 12 h light/dark cycle and had free access to standard pellet diet and water. Prior to the experiment, all rats were allowed one week to acclimatize to the laboratory environment. All experimental procedures involving animals were reviewed and approved by the Research Ethics Committee of faculty of pharmacy, MSA University. A total of 25 rats were randomly assigned to five experimental groups ($n = 5$ per group). The negative control croup received 1 mL of saline orally by gavage each day for 14 days and a rectal dose of 0.5 mL saline on day 13. The positive control group (induction group) followed the same oral saline regimen but received a rectal dose of 0.5 mL of 4% (v/v) acetic acid on day 13 to induce colitis. The commercial probiotic group received a daily oral dose of 1 mL of a commercial probiotic preparation containing 2 billion CFU per 5 mL for 14 days, followed by rectal administration of 4% acetic acid on day 13. The novel *Lactiplantibacillus plantarum* C4 Group was treated in the same way as the commercial probiotic group but with the novel bacterial strain instead, *Lactobacillus* was cultured in MRS broth in anaerobic condition for 24 h, cell pellet was washed twice and suspended in normal saline and the CFU/mL concentration is adjusted to 4×10^8 CFU/mL by measuring optical density (OD600nm) verified by viable count on MRS agar plate. Finally, the standard drug treated group received 1 mL of saline orally for the first 7 days, followed by daily oral administration of sulfasalazine at a dose of 50 mg/kg body weight from day 8 to day 14, and also received 1mL of 4% acetic acid rectally on day 13. This experimental setup allowed for the comparison of the protective or therapeutic potential of the tested treatments against acetic acid-induced colitis.

2.3 Samples collection and storage

At day 14 the animals were sacrificed by cervical dislocation under prior anesthesia by Intraperitoneal (IP) injection of ketamine/xylazine mixture (90/10 mg/kg respectively). The colon was collected and washed thoroughly with saline then the length and weight were measured accurately, then a part of the distal colon was preserved in formalin for histological analysis. Another parts of the colon were homogenized, using rotor stator

homogenizer, in tris buffer (pH 8.5) or in phosphate buffered saline of pH 7.4 (1gm per mL), tissue homogenates were stored at -80 °C for biochemical analysis by ELISA.

2.4 Histopathological examination

Samples of the distal colons were fixed immediately in 10% formaldehyde, embedded in liquid paraffin, cut into transverse sections of 5 μm thick using a Leica RM 2125 Microtome (Leica Biosystems, Wetzlar, Germany), and then mounted on glass slides and stained with haematoxylin and eosin (H&E). Microscopic changes such as necrosis, fibrosis, hyperemia, epithelial damage, ulceration, infiltration, and submucosal abscesses were scored on a 0–4 scale where 0 denotes no detectable damage and 4 denotes most severe damage.

2.5 ELISA determination of inflammatory mediators

ELISA plates were pre-coated with an antibody specific to Rat IL-6, IL-1β or TNF-α. After adding samples/standards, a biotinylated detection antibody specific for the biomarker to be measured and Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each well and incubated for 1 h at 37 °C then the substrate was added and incubated with the mixture for 15 min at 37 °C before the addition of stop solution. The optical density of the product was measured spectrophotometrically at a wavelength of 450 nm using a plate reader (BMG Labtech, FLUOstar Omega, Germany). The OD value is proportional to the concentration of the measured biomarker.

2.6 Enzyme activity determination

2.6.1 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using the activity assay kit from sigma Aldrich. The activity of the enzyme is calculated depending on the interference of superoxide dismutase with the reduction of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt (SWT-1) to produce SWT-1 formazan. So the activity of the enzyme is calculated as percentage of inhibition of the production of SWT-1 formazan by subtraction of the absorption of sample from the absorption of the blank at 450 nm using UV/Visible Spectrophotometer Jenway 6305 (USA). 0.5 g of tissue was homogenized in 5 mL of 50 mM potassium phosphate buffer, pH 7.4 with 0.1 mM EDTA and 0.5% triton X-100. Tissue homogenate was then centrifuged at 12,000 rpm and supernatant was used for SOD activity determination as described in kit manufacturer manual.

2.6.2 Catalase activity

The activity of catalases enzyme in sample is proportional to the decrease in the hydrogen peroxide externally added to the sample. The activity was determined by the addition of 500 μL of H_2O_2 solution in tris buffer (pH 8.5) to 100 μL of tissue homogenate supernatant and let them to react in 37 °C for 10 min, then 1:3 mixture of 5% potassium dichromate and glacial acetic acid was added to reaction mixture (1:1) to stop the enzymatic reaction and to react with the remaining H_2O_2. The produced color was measured at 620 nm and the enzyme activity was calculated by the difference in the absorption between the samples and the blank.

2.7 Malondialdehyde level

Malondialdehyde level was determined for the assessment of lipid peroxidation level. MAD reacts with thiobarbituric acid to produce colored product measured at 532 nm. The kit procedure has been followed, briefly 10 mg of tissue was homogenized in lysis buffer, centrifuged then thiobarbituric acid has been added and the mixture incubated at 95 °C for 60 min. The mixture has been cooled and absorbance has been measured.

2.8 Statistical analysis

Five samples from each group were analyzed for each tested parameter. All analyses were done in triplets for each sample and mean and SD were calculated. GraphPad Prism 5 was used to perform statistical analysis. Kolmogorov Smirnov test was performed to check data normality and one-way ANOVA test was used to test if there is a significant difference between the test groups, Tukey's post hoc test was used to determine the groups showing different values from the other groups if any. The difference was considered significant if the p-value was < 0.05.

3 Results

3.1 Histopathological examination

Histopathological examination showed normal tissues with no histological alternation in control group. Histological structure of the mucosa with lamina propria and glands as well as the underlying submucosa, muscularis and serosa were normal (Fig. 1A). The induction group has showed focal massive inflammatory cells infiltration in the lamina propria of the mucosa associated with edema and congestion in the blood vessels of the submucosa (Fig. 1B). All the protected groups showed histological improvement compared to the induction group. The groups protected by our candidate probiotic strain as well as the group protected by sulfasalazine showed no histological alternation as shown in Fig. 1C for candidate probiotic strain protection and in Fig. 1D for sulfasalazine

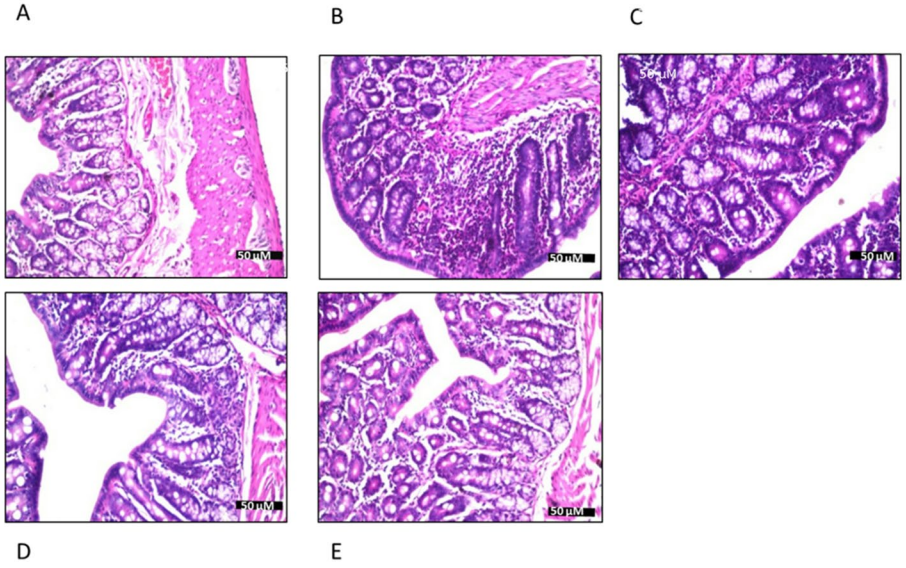

Fig. 1 Transverse sections in distal colon of test groups stained with haematoxylin and eosin. (A) represents the negative control (no induction or treatment). (B) represents the positive control (induction by acetic acid only), (C, D and E) represent groups exposed to induction of IBD by acetic acid and received protection by either the novel candidate probiotic strain or sulfasalazine or Enterogermina respectively. Scale bar = 50 µm

protection. Finally, the group protected by Enterogermina preparation showed mild inflammatory signs represented in mild edema with few inflammatory cells infiltration in the lamina propria of the mucosa associated with edema in the muscular layer (Fig. 1E).

3.2 Effect of novel probiotic strain on inflammatory markers level

Inflammatory markers, IL-6, IL-1β and TNF-α were determined in colon tissues. The level of all studied inflammatory biomarkers showed significant increase in the group exposed to inflammation induction by acetic acid without any treatment or prophylaxis; IL-6 has been increases from 37.7 ± 3.03 to 295.4 ± 25.58 pg/mL which is about 8 folds increase, IL-1β has also showed an increase from 379.5 ± 42.07 in control group to 1756 ± 89.66 pg/mL in induction group by more than 4 folds increase, TNF-α has also increased by around 7 folds from 491.8 ± 31.81 to 3593 ± 354.0 pg/mL. Treatment and prophylactic interventions with sulfasalazine, Enterogermina, and the novel *Lactiplantibacillus plantarum* C4 strain significantly reduced the elevated cytokine levels. IL-6 concentrations were reduced to 44.18 ± 5.88, 119.8 ± 21.30, and 41.24 ± 3.72 pg/mL in the sulfasalazine, Enterogermina®, and *Lactiplantibacillus plantarum* C4 groups, respectively. Corresponding reductions were also observed for IL-1β (464.9 ± 43.18, 620.2 ± 52.64, and 431.6 ± 44.78 pg/mL) and TNF-α (362.2 ± 46.64, 734.5 ± 74.90, and 477.5 ± 47.95 pg/mL) in the same respective groups. Both sulfasalazine and the tested probiotic strain were able to decrease the inflammatory markers to the normal level as proven by the statistical analysis, ANOVA and Tukey's post hoc test. Enterogermina also showed significant improvement in the level of the inflammatory markers compared to the induction group however the levels of IL-6 and IL-1β were still significantly higher than the control group. The results of ELISA determination of the inflammatory markers, IL-6, IL-1β and TNF-α in colon tissues of the studied groups are shown in Fig. 2A, B and C respectively.

3.3 Effect of novel probiotic strain on SOD and catalase activity

The induction of colitis using acetic acid resulted in a marked reduction in the activity of antioxidant enzymes in colon tissues. SOD activity was decreased by approximately 65%, from 18.07 ± 1.19 to 6.15 ± 0.73 U/10 mg of tissue. Similarly, catalase activity was

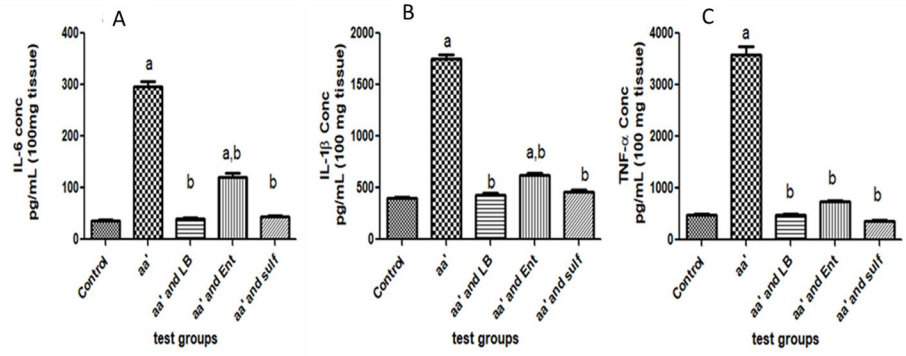

Fig. 2 Levels of inflammatory biomarkers in test groups. Control, aa (induction by acetic acid), aa' and LB (induction and treatment by tested probiotic strain), aa' and Ent (induction and treatment by Enterogermina), aa' and sulf (induction and treatment by sulfasalazine). (A, B and C) show levels of IL-6, IL-1β and TNF-α respectively. [a] significantly different from control, [b] significantly different from acetic acid group, $p < 0.05$.

dropped by about 73%, from 15.28 ± 1.15 to 4.73 ± 0.99 U/10 mg of tissue. Treament with Enterogermina partially restored antioxidant enzyme activity. SOD activity improved to 12.8 ± 2.22 U/10 mg, representing a recovery of approximately 50% of the lost activity. Catalase activity increased to 13.74 ± 1.59 U/10 mg, restoring nearly 88% of the lost function. Treatment with sulfasalazine or the tested probiotic strain almost completely restored the activity of both enzymes. SOD activity returned to 17.7 ± 0.68 and 17.4 ± 1.19 U/10 mg, while catalase activity was restored to 14.8 ± 1.54 and 13.94 ± 1.06 U/10 mg in the sulfasalazine and tested probiotic groups, respectively. Figure 3A and B show the activity of SOD and catalase in test groups, respectively.

3.4 Effect of novel probiotic strain on malondialdehyde level

Malondialdehyde level has been greatly increased in colon tissues from 5.24 ± 2.32 to 63.86 ± 9.23 nmol/10 mg tissue due to the induction of colitis by acetic acid indicating great increase of lipid peroxidation. The level has been significantly decreased by the treatment using sulfasalazine, Enterogermina and the novel probiotic strain. Sulfasalazine and the novel probiotic treatment have returned the peroxidation level to the normal level, 5.82 ± 1.68 and 10.84 ± 3.35 nmol/10 mg respectively. Enterogermina has decreased the level of malondialdehyde to 13.04 ± 3.78 nmol/10 m, g which is significantly less than the induction group, however it was still also significantly higher than the control group level. Figure 4 shows the results of malondialdehyde level in test groups.

4 Discussion

While the exact cause of inflammatory bowel disease is still unclear, research increasingly shows that imbalances in gut microbiota play a key role. Changes in intestinal bacteria can weaken the gut barrier, increase inflammation, and affect immune responses, making the host more prone to disease [20, 21]. This has led to interest in using probiotics as a treatment and prevention strategy for IBD. Probiotics are appealing due to their safety, ease of use, and potential for long-term application. Studies suggest that, probiotics help reduce inflammation by lowering pro-inflammatory signals, supporting

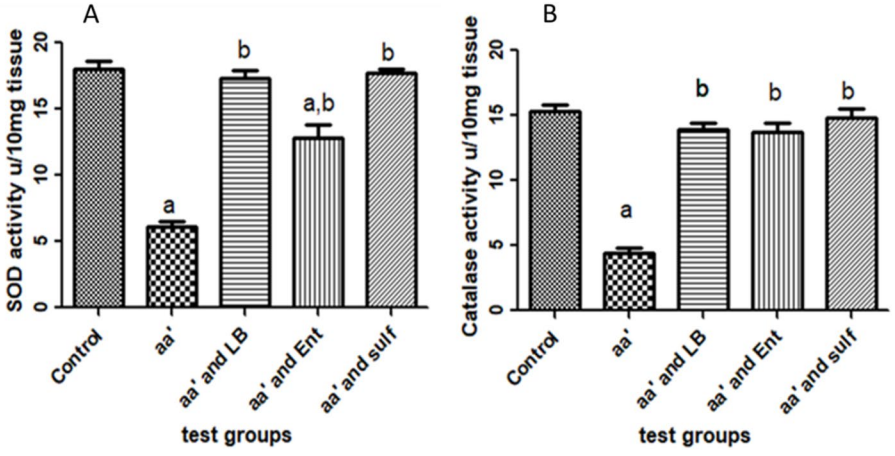

Fig. 3 Activity of SOD and catalase enzymes in colon tissue of test groups, Control, aa' (induction by acetic acid), aa' and LB (induction and treatment by new probiotic strain), aa' and Ent (induction and treatment by Enterogermina), aa' and sulf (induction and treatment by sulfasalazine). (A and B) show the activity of SOD and catalase enzymes respectively. [a] significantly different from control, [b] significantly different from acetic acid group, $p < 0.05$

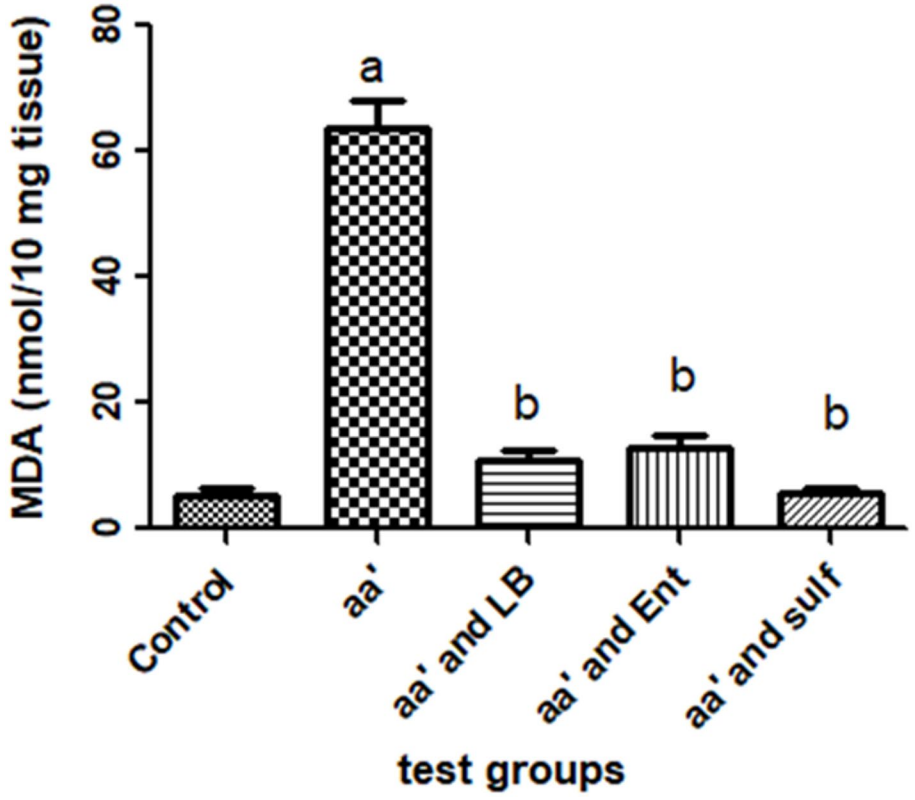

Fig. 4 Level of Malondialdehyde in colon tissue of test groups, Control, aa (induction by acetic acid), aa' and LB (induction and treatment by new probiotic strain), aa' and Ent (induction and treatment by Enterogermina), aa' and sulf (induction and treatment by sulfasalazine). [a] significantly different from control, [b] significantly different from acetic acid group, $p < 0.05$

anti-inflammatory processes, and strengthening the intestinal lining [17]. They also fight harmful bacteria and may improve disease outcomes. Although many studies support their effectiveness, identifying the most beneficial strains remains a major research goal. Among the most promising are Bifidobacterium and Lactobacillus species, which have shown strong therapeutic potential in IBD [22, 23].

In this study, a promising *Lactiplantibacillus plantarum* probiotic strain has been assessed for IBD treatment in rats. The candidate *Lactiplantibacillus plantarum* C4 strain has been isolated during a screening project for isolation, identification and characterization of several lactobacillus strains [24]. More than 50 *Lactobacillus* strains were isolated from different sources including cow milk, dairy products and even infants stool. The isolates have identified to genus level by microscopical examination and biochemical tests: Gram staining (Gram positive bacilli, non-endospore forming), catalase negative, and oxidase negative. Identification of the probiotic strain to the species level was carried using API CHL 50 system (Biomerieux, Marcy l' Etoile, France), a standardized system consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms. The isolates were tested for their acid and bile salt tolerance. The selected strain in this study has showed prominent tolerance to acidic condition and bile salts which make it a good candidate for treatment of GIT conditions.

The results of this study showed the superior efficacy of the candidate probiotic strain against IBD induced by acetic acid in rats. Histopathological examination showed the prophylactic and curative effect of tested probiotic strain which is comparable to effect

of sulfasalazine effect and better than the standard probiotic formula. The histopathological results are completely consistent with the biochemical analysis of inflammatory biomarkers in colon tissues; the biomarkers levels were prominently increased in the induction group while the treatment with the new probiotic strain restored the inflammatory markers level to almost the normal values, this is most probably due to the prevention of the infiltration of inflammatory cell to the tissues which was obvious in the histopathological examined fields. The nuclear factor kappa B (NF-κB) signaling pathway, which is influenced by the JAK/STAT pathway, plays a key role in controlling the production of various inflammatory molecules [25, 26]. Recent studies have shown that certain *Lactobacillus* strains can influence the JAK/STAT pathway [27], which may explain their ability to lower the levels of inflammatory mediators. In addition, NF-κB and other inflammatory pathways can be affected by oxidative stress [28].

Several studies have highlighted the link between oxidative stress and inflammation in IBD and other conditions [29]. Oxidative stress is believed to actively contribute in the development of IBD in different mechanisms including the recruitment of immune cells, activation of inflammatory pathways, alternation of cell membrane of colon epithelia and activation of NF-κB pathway. Our results are compatible with these facts as the induction group has shown a significant increase in the oxidative stress in colon tissue represented in the significant decrease in SOD and catalase activity combined with the high level of MAD as expected. The candidate probiotic strain has also showed significant positive effect regarding the oxidative stress. The activity of enzymes was restored to the normal level in the groups protected with the candidate strain and sulfasalazine. The level of MAD was also kept in the treated groups as low as the control group reflecting the significant protective effect of the tested treatments against the oxidative stress effect on cell membranes.

All the results of histopathology, inflammatory markers and oxidative stress measurements have showed the relatively superior protective effect of the new *Lactiplantibacillus plantarum* C4 strain in the treatment of IBD. The new strain showed protective effect which is stronger than the commercial strain and similar to one of the most effective drugs for IBD. Taking in the consideration the serious side effects of the medication and the high safety and the easiness of probiotic administration as food additive or supplement, the use of the candidate probiotic in the treatment of IBD could be recommended. The features of the candidate strains, low pH and bile salt tolerance [24], could help to maximize their desirable effects in the GIT.

The effect of the candidate strain is probably due to their protective effect against some pathogens [19] or due to improving the tight junction between the epithelial cells in GIT lining. Other possible reasons are the immunomodulatory and anti-inflammatory effect. Despite the very promising results of this study it has some limitations represented in the employment of one animal model where colitis is induced using only chemical induction which mimics ulcerative colitis features but does not fully replicate the complex etiology of human IBD. The findings may not necessarily extend to other IBD models as IBD may be induced due to variable chemical, biological and even genetic factors. Also this study has only evaluated the short term effect of the treatment while long-term effects of the probiotic intervention were not assessed. Chronic models or extended follow-up studies are needed to evaluate the durability of the therapeutic effect. In addition, the exact mechanisms and pathways of the curative and protective effect of

the candidate probiotic strain are not fully identified. Some further investigations are required to investigate the precise mechanism and to describe the interaction between IBD patient gut microbiota and the novel teste probiotic strain. Also dose dependent effect and the effect of the candidate probiotic strain in combination with another treatment approaches need to be investigated in further research.

In conclusion, the tested *Lactiplantibacillus plantarum* C4 probiotic strain has prominent protective effect against chemically induced IBD in rats. The tested strain decreases the infiltration of inflammatory cells and decreases the level of inflammatory markers and oxidative stress. Further research is required to discover the full precise molecular mechanism of action of the candidate probiotic strain against IBD.

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Author contributions
A.S and A.A equally contributed in formal analysis and paper writing and review. A.A had the major contribution in the research concept. A.S has the major contribution in building the experimental design.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the institutional animal care and use committee of faculty of pharmacy, October University for Modern Sciences and Arts (MSA-Faculty of pharmacy IACUC) (approval ref PB22/REC22/2024PhD). All animal experiments comply with Guide for the care and use of laboratory animals, 8th Edition 2011, by National Research Council (US).

Competing interests
The authors declare no competing interests.

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