



Beneficial Effect of Synbiotic Combination of *Limosilactobacillus fermentum* FS-10, *Lactiplantibacillus plantarum* Lp1-IC and Short-Chain Fructooligosaccharides in Colitis Murine Model

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Abstract

Therapies targeting gut microbiota are being extensively researched for colitis patients. In this study, we have tested the efficacy of indigenously isolated strains *Lactiplantibacillus plantarum* Lp1-IC and *Limosilactobacillus fermentum* FS-10 and their combination with short-chain fructooligosaccharides (sc-FOS) in mice models of DSS-induced colitis. For a desired efficacy, a synbiotic should be very meticulously formulated with the right choice of prebiotic and probiotic. Therefore, the ability of lactobacilli to utilize scFOS for growth was first tested by culturing the strains in a specially designed minimal media supplemented with scFOS as carbon source. The bacteria utilized scFOS and produced metabolites such as acetate and lactate. Thereafter, the in vitro anti-inflammatory effect was tested on markers such as TNF-alpha (TNF- α), nitric oxide and IL-10 in human monocyte (THP-1) and mouse macrophage (Raw 264.7) cell lines. The in vivo efficacy was studied in mice model of DSS-induced colitis, and the effect on the systemic and localized inflammatory markers was assessed in serum and colon tissue samples respectively. Administration of DSS elicited predominant clinical signs of weight loss, diarrhoea, faecal occult blood, increase in inflammatory markers and extensive damage of colon tissue. These symptoms were significantly reversed in all the treatment groups; however, the combination of lactobacilli and scFOS performed better than the individual ingredients. The study highlights the potential of the indigenous lactobacilli strains, scFOS and their combination for management of gut inflammation in colitis patients.

Keywords Lactobacillus · Synbiotic · Short-chain fructooligosaccharide · Inflammation · Colitis

Introduction

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD), and the underlying cause or the aetiology of the disease is still unclear. A disturbed gut microbiota has been attributed as the major contributing factor in the development of UC. It is a chronic inflammatory condition, and studies have revealed the occurrence of dysbiosis due to an abundance of pro-inflammatory microbiota such as *Escherichia coli*, *Ruminococcus gnavus* or *Fusobacterium* [1]. Therefore, therapies aimed at reversing dysbiosis by creating an abundance of anti-inflammatory microbiome through administration of probiotics and prebiotics are evolving.

Probiotics are live microorganisms that are beneficial for health. Studies have attempted to explore the efficacy of probiotics for treatment of UC. The anti-inflammatory properties of lactobacilli and bifidobacilli have been exploited for management of gut-associated inflammation in colitis. VSL#3, a proprietary mixture of strains of *Lactobacillus*, *Bifidobacterium* and *Streptococcus salivarius*, induced remission in UC patients [2]. *Lactobacillus rhamnosus* GG (LGG) is a well-documented probiotic exhibiting beneficial effects such as prevention of gastrointestinal infection and diarrhoea, and it is also known to stimulate the immune response. Therefore, in the current study, *Lactobacillus rhamnosus* GG (LGG) is used as a positive control or benchmark [3].

Prebiotics, such as inulin, oligofructose, beta glucans and galactooligosaccharides, selectively promote the adhesion and proliferation of beneficial bacteria residing in the gut. These prebiotics exert a direct or indirect impact on the gut and the immune system. They can directly bind to the epithelial cells and accelerate the healing process to restore the

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damaged epithelium. Indirectly, they increase the production of SCFAs (acetate, butyrate and propionate) that helps in the proper functioning of the intestine [4]. Numerous studies have documented the beneficial role of prebiotics on the gut microbiota. Prebiotics have also been shown to reduce the production of inflammatory cytokines, such as IL-1 α , IL-1 β , IL-6, IL-12, TNF- α and IFN- γ , and increase the expression of intestinal tight junction proteins that help in maintaining the gut barrier integrity. Due to their ability to selectively promote the beneficial microflora, the prebiotics decrease the population of pathogenic microorganisms that induce the production of inflammatory factors. Thus, prebiotics indirectly create an anti-inflammatory microenvironment that renders them as potential candidates for IBD management [5].

Combination of probiotics with the correct prebiotics or synbiotics may provide the added advantage of promoting survival and colonization of the probiotic strain in the gut and improve the resident flora of the gut. Prebiotics are largely fermented by anaerobic bacteria residing in the colon that produce SCFA, CO₂ and H₂ resulting in the drop of the intraluminal pH favouring the growth of lacto- and bifidobacilli. The SCFA also exhibits immunoregulatory properties and helps in the management of inflammatory response [6, 7]. Synbiotics hold promise to be explored as a therapeutic option in gut inflammatory disease. However, for designing a synbiotic formulation, it is important to select the appropriate combination of prebiotic and probiotic for a synergistic effect. In this study, we have designed and tested a synbiotic formulation comprising indigenous isolated strains of *L. plantarum* and *L. fermentum* and short-chain fructooligosaccharide in mice model of colitis. We have also tested the individual efficacy of the probiotic and prebiotic when used in isolation.

Materials and Methods

Isolation and Culture of *Lactobacillus*

Two lactobacillus species, *L. fermentum* FS-10 [8] and *L. plantarum* Lpl-IC, isolated from fermented cabbage or sauerkraut were used in the study. The process of cabbage fermentation involves removal of the outer leaves followed by washing and chopping of 200 g of cabbage. Three per cent w/v of NaCl (Merck) was added, the mixture kneaded and kept at ambient temperature for 15 days. After the 15 days of fermentation, 1 ml of the released juice was collected aseptically and serially diluted in sterile saline dilution (0.85% NaCl) and plated on MRS agar and incubated at 37 °C for 24 h. Morphologically distinct colonies were selected. The selected colonies were purified by repeated streaking on the appropriate agar media and sub-cultured

periodically for further studies. The suspected lactobacillus species were further cultured for morphological identification and 16 s rRNA sequencing. *L. plantarum* Lp1-IC strain was identified based on the 16 s rRNA sequence. The strain was qualified as a potential probiotic using various in vitro test for pH tolerance (S1), adhesion (S2) and toxicity (ST1 and ST2). The probiotic attributes of *L. fermentum* FS-10 have been published previously [8]. *L. rhamnosus* GG (53,103) was procured from ATCC and revived in MRS media.

The GenBank accession numbers of the sequences are OR516801 and OQ255609 for *L. plantarum* Lp1-IC and *L. fermentum* FS-10 respectively.

Prebiotic Short-Chain Fructooligosaccharide

scFOS is manufactured by Tata Chemicals Limited under the brand name FOSSENCE®. It is produced through bio-transformation from sucrose.

Growth Kinetics in Minimal Media and Metabolite Analysis

The *L. fermentum* and *L. plantarum* strains were revived in MRS media and allowed to grow for 24 h. The strains were then transferred to a specially designed media with reduced carbohydrate (0.5% scFOS) and nitrogen source (Table 1). Media supplemented with 0.5% glucose was used as control. The bacterial suspension in minimal media was added to a 96-well plate, and absorbance was measured every hour until 24 h using a multimode reader.

For metabolite identification, the lactobacilli were grown in minimal media containing scFOS as carbohydrate source for 24 h at 37 °C. The cell suspension was centrifuged and the supernatant collected and subjected to HPLC analysis.

Table 1 Half-strength MRS with scFOS as sole carbon source

S. No	Ingredient	g/Lit
1	scFOS	5
2	Ammonium citrate	2
3	Di-potassium phosphate	2
4	Polysorbate 80	1
5	Proteose peptone	0.5
6	Yeast extract	0.25
7	Magnesium sulphate	0.1
8	Manganese sulphate	0.05

Cell Culture and Generation of Conditioned Cell-Free Supernatants

Human monocytic cell line THP-1 and mouse macrophage cell line RAW264.7 were procured from the National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 (Gibco) and DMEM (Gibco), respectively, at 37 °C in 5% CO₂ and 95% humidified air. The cell culture media were supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 µg/ml streptomycin and 100 U/ml penicillin. The THP-1 cells were differentiated to macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) (2 ng/ml) for 24 h followed by activation with bacterial LPS (1 µg/ml) (Sigma Aldrich) for 24 h.

The conditioned cell-free supernatants (CFS) were generated by growing the bacteria in glucose-free DMEM media supplemented with 0.5% scFOS using the method described previously [8]. This conditioned media is enriched with bacterial metabolites and has been previously shown to possess anti-inflammatory property [8].

Cytokine ELISA and Nitric Oxide Assay

THP-1 cells were seeded in a six-well plate at density of 1×10^6 cells/well and allowed to grow for 24 h. The cells were then differentiated into macrophage-like cells with combination of PMA and LPS. The cells underwent a distinct morphological change upon differentiation resembling monocyte-derived macrophages. The conditioned CFS was added at a final concentration of 12% in each well and incubated for 24 h. The THP-1 macrophages cultured in DMEM media supplemented with 0.5% scFOS were used as control. The levels of TNF- α and IL-10 in the THP-1 supernatants were evaluated using ELISA kit (BD Bioscience), according to the manufacturer's instructions.

Nitric oxide levels were estimated in Raw 264.7 cell line. The cells were seeded in a six-well plate at density of 1×10^6 cells/well and stimulated with LPS. The conditioned CFS was added at concentration of 12% v/v per well. The LPS-activated cells grown in DMEM media with 0.5% scFOS without CFS were used as control. The nitric oxide levels were estimated using a nitric oxide estimation kit (Sigma Aldrich), as per the manufacturer's instructions.

Animals

C57-BL-6 male mice (originally from Charles River Laboratory) were housed in an animal room having controlled temperature (22 °C $\pm$ 3 °C) and humidity range (30–70%). The animals were provided with 12-h light and 12-h darkness and housed in IVC systems. Feed was sourced from SDS diet services, and autoclaved drinking water was provided ad libitum. This study was performed as per ethical practices

laid down in the CPCSEA guidelines for animal care and use. The study was approved by the Institutional Animals Ethics Committee (IAEC) of the test facility (TheraIndx Pvt. Ltd, Bangalore, India).

DSS-Induced Colitis

Colitis was induced by feeding DSS 2.5% in drinking water. Fifty grams of DSS was weighed and dissolved in 2 l of sterile drinking water to achieve the desired concentration of 2.5%.

Preparation of *Lactobacillus* Powder

Bacteria were grown in a 50 l fermenter maintained at 37 °C for 24 h. The biomass was collected by centrifugation, mixed with skim milk powder and sucrose and freeze dried into a powder. The viability of the probiotic was > 95% post freeze drying.

Treatment Groups and Procedure

The mice were randomly divided into 10 groups ($n=8$): normal (no treatment), DSS treatment, DSS + vehicle control (skim milk and sucrose 10 mg/day), DSS + scFOS (75 mg/day), DSS + probiotics (three probiotic treatment groups; all 1×10^7 CFU/day), DSS + probiotic + scFOS (three synbiotic-treated group 75-mg scFOS + 10^7 CFU/ml of lactobacilli). During the experiment, the animals were administered the required quantity of test sample suspended in 25 µl of sterile saline once a day by oral gavage. Experimental colitis was induced after 8 days of therapy by adding 2.5% DSS (MP Bio-medicals, LLC) to the drinking water for 7 days, i.e. from day 8 to day 14. On day 15, DSS was replaced with sterile drinking water for the rest of the treatment duration. On day 21, the mice were euthanized; the blood and colon were isolated for further analyses. The outline of study design has been incorporated in Table 2. The body weight and stool consistency are recorded.

DAI Scores

The Disease Activity Index (DAI) score was calculated on the basis of body weight, stool consistency and blood in the stool during the experimental period using the formula.

$$\text{DAI} = (\text{weight loss score} + \text{stool consistency score} + \text{bloody stool score})/3$$

The scoring criteria are mentioned in Table 3.

Serum Cytokine Estimation by ELISA

Blood was collected on day 15 (non-terminal, 0.2 ml of blood was drawn from saphenous vein) and day 21 (terminal collection through cardiac puncture). Serum was harvested and used for

Table 2 Study design

	Week 1	Week 2 (no DSS phase)	Week 3 (DSS phase)	Week 4 (post DSS phase)
Normal control	Acclimatization	Saline	Saline	Saline
Disease control	Acclimatization	Saline	DSS + Saline	Saline
Disease treated	Acclimatization	VC scFOS Probiotics Synbiotics	DSS + VC DSS + scFOS DSS + Probiotics DSS + Synbiotics	VC scFOS Probiotics Synbiotics

measurement of TNF- α , IL-1 β , IL-17, IL-6 and IL-10 by using commercially available ELISA kits (Krishgen BioSystems).

Colon Marker Expression by Real-Time PCR Analysis

The colon tissues were removed aseptically, weighed and homogenized in saline. RNA was isolated from the homogenized tissue (Nucleo-pore® RNASure® Mini Kit) and quantified using TECAN-Nano Quant Plate™. The expression levels of TNF- α , IL-1 β , IL-17, IL-6 and IL-10 were estimated by one-step SYBR Green Quantitative Real-time PCR (qRT-PCR). RT-PCR reaction was carried out using RNA-direct™ SYBR® Green Realtime PCR Master Mix Cat#QRT-20 kit and analysed on a QuantStudio™ 3 Real-Time PCR System. The sequence of primers used in the study are listed in Table 4. The transcription level of the genes of interest was determined using $2^{-\Delta\Delta C_t}$, where ΔC_t is the difference between the C_t values of the target gene and the GAPDH reference gene. The fold change in mRNA level for the test group is expressed relative to the DSS group.

Histopathology

The length of the colon was measured prior to processing (S3). A part of the colon tissue was processed for preparation of paraffin blocks. Blocks were sectioned to 3 to 5 microns using rotatory microtome and mounted on clean glass slides. The slides were stained with haematoxylin–eosin (H&E) stain and observed under a light microscope. The histological score was assessed by a professional pathologist who was blinded to the study.

Table 3 DAI score indicator

Score	Weight loss (%)	Stool consistency	Bloody stools
0	None	Normal	None
1	1–5	Soft and shaped	Between
2	6–10	Loose	Faecal occult blood
3	11–15	Between	Between
4	> 15	Diarrhoea	Defecate haemorrhage

Histological scores were assigned in parallel as follows: 0 = normal tissue, 1 = superficial epithelial damage, 2 = focal ulcer confined to the mucosa, 3 = focal transmural inflammation and ulceration, 4 = extensive transmural ulcer and inflammation, normal mucosa between lesions, 5 = extensive flaky transmural ulcers and inflammation.

Data Analysis

All data was expressed as the mean $\pm$ standard deviation. The significance of differences between the control and treatment groups was evaluated using a one-way ANOVA at 95% confidence level in GraphPad Prism. A P value of < 0.05 will be considered as a significant point.

Results

Growth and Metabolite Production of Lactobacilli in Minimal Media Supplemented with scFOS as Carbohydrate Source

The growth kinetics of various lactobacilli strains were studied for 24 h in a specially designed minimal medium having scFOS as the source of carbohydrate. The absorbance was

Table 4 Sequence of gene primers used in the study

Gene	Primers
M-GAPDH-FP	TGACCTCAACTACATGGTCTACA
M-GAPDH-RP	CTCCCCATTCTCGGCCTTG
M-IL-6 FP	GGCGGATCGGATGTTGTGAT
M-IL-6 RP	GGACCCCAGACAATCGGTTG
M-TNF- α FP	GGTGCCTATGTCTCAGCCTCTT
M-TNF- α RP	GCCATAGAACTGATGAGAGGGAG
M-IL-1 β FP	TGGACCTTCCAGGATGAGGACA
M-IL-1 β RP	GTTCATCTCGGAGCCTGTAGTG
M-IL-17 FP	CAGACTACCTCAACCGTTCCAC
M-IL-17 RP	TCCAGCTTTCCTCCGCATTGA
M-IL-10 FP	CGGGAAGACAATAACTGCACCC
M-IL-10 RP	CGGTTAGCAGTATGTTGTCCAGC

recorded every hour in a multimode reader. Both strains could utilize scFOS for growth. Amongst the two strains, the growth of *L. plantarum* strain was higher than the *L. fermentum*. *L. rhamnosus*, on the other hand, exhibited restricted growth in the presence of scFOS compared to glucose (Fig. 1A). The metabolite analysis also exhibited a similar profile. *L. fermentum* and *L. plantarum* could utilise scFOS and produce lactic and acetic acid, whereas *L. rhamnosus* did not show increase in metabolite production in the presence of scFOS against glucose (Fig. 1B).

In Vitro Anti-inflammatory Activity in Presence of scFOS

The in vitro anti-inflammatory activity of lactobacilli was assessed by determining the effect of conditioned cell-free supernatant (CFS) of *L. fermentum* FS-10, *L. plantarum* Lpl-IC and *L. rhamnosus* GG on pro-inflammatory markers such as TNF- α , NO and anti-inflammatory IL-10. LPS-stimulated THP-1 macrophages were exposed to CFS, and the effect on TNF- α and IL-10 levels was analysed. The Raw 264.7 cells were stimulated with LPS to induce production of NO, and the effect of the CFS was estimated. The CFSs exhibited

differential response in modulation of levels of TNF- α , IL-10 and NO (Fig. 2). The *L. fermentum* and *L. plantarum* supernatants very significantly downregulated TNF- α levels. The NO level was significantly downregulated, and IL-10 was upregulated by *L. fermentum* CFS. The control cell exposed to 0.5% scFOS-containing media has been used as control to record the baseline response of cells. The findings indicate the potential of *L. fermentum* and *L. plantarum* to act in synergy with scFOS. This prompted further study in colitic mice model to understand the in vivo efficacy of the combination of probiotics and scFOS.

Effect on Body Weight and Disease Activity Index

Body weights were measured daily before vehicle and test item administration. After 7-day induction with 2.5% DSS, reduction in feed consumption, change in stool consistency and decrease in body weights of the mice indicated successful model establishment. Body weight loss was observed in all the groups from day 8 to day 14. At the end of the study, significant body weight gain ($p < 0.05$) was observed in all treatment groups when compared to DSS control (Fig. 3A).

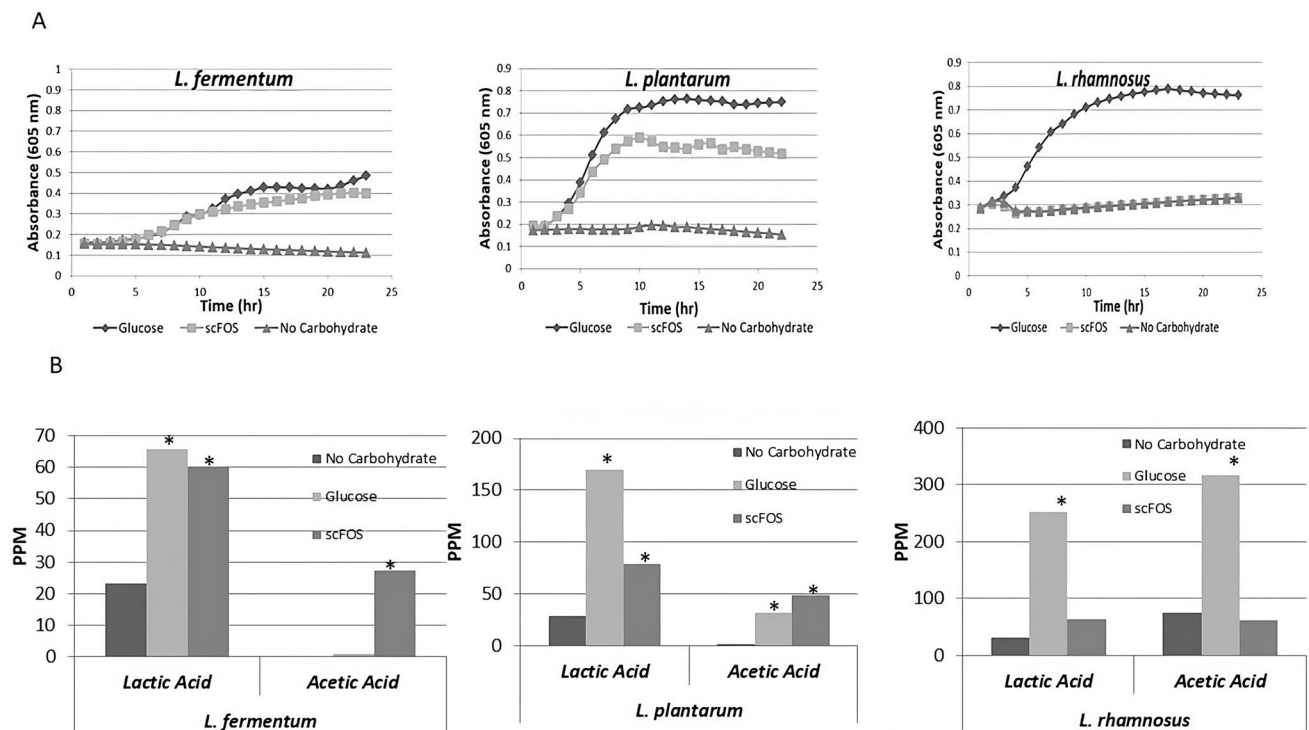


Fig. 1 Growth and metabolite production of lactobacilli in minimal media having scFOS or glucose as carbohydrate source. **A** Growth kinetics of *L. fermentum*, *L. plantarum* and *L. rhamnosus* in a specially designed minimal media having scFOS or glucose as carbohydrate source. The graphs depict the hourly growth pattern of the strains up to 24 h. The x-axis displays absorbance and the y-axis dis-

plays time. **B** Lactic acid and acetic acid produced by fermentation of glucose or scFOS by lactobacilli. The x-axis represents the levels of acetic or lactic acid in parts per million (ppm). * $p < 0.05$ significant difference compared to metabolite production in absence of carbohydrate source

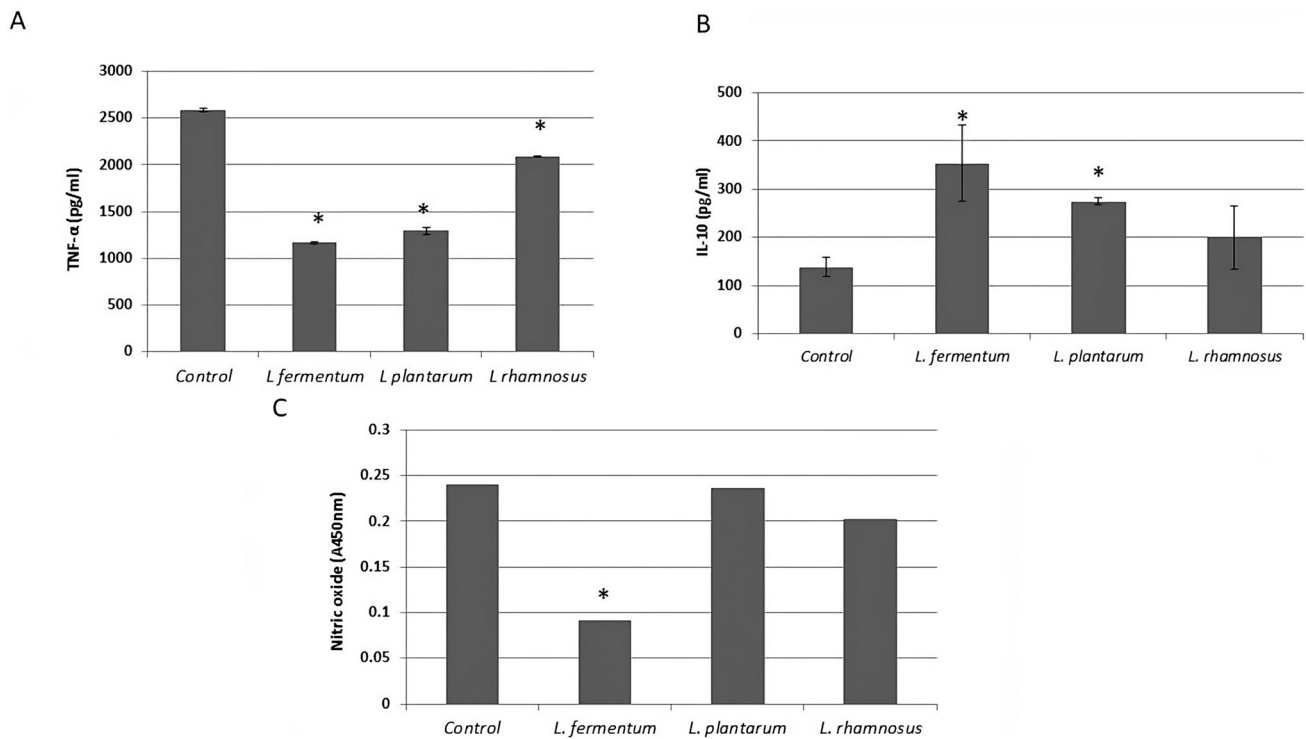


Fig. 2 In vitro anti-inflammatory effect of lactobacilli in presence of scFOS. The LPS-stimulated THP-1 cells (**A**, **B**) and Raw 264.7 (**C**) were exposed to bacterial CFS generated in presence of scFOS. Levels of TNF-α (**A**), IL-10 (**B**) and nitric oxide levels (**C**), compared

to control (LPS-treated cells exposed to 0.5% scFOS) were observed. The data is represented as mean ± SD of three independent experiments. * $p < 0.05$ statistically significant difference compared to control

DAI was recorded during the entire experimental period. DAI was calculated from the percentage loss in body weight in combination with stool consistency and bloody stools. The DAI score increased significantly after DSS intake, in DSS control [2.5% DSS + saline] and

vehicle groups, while it was markedly attenuated in the treatment groups (Fig. 3B). Treatment groups reduced the appearance of the colitis symptoms like occurrence of hemocult and diarrhoea which resulted in a significant reduction of DAI.

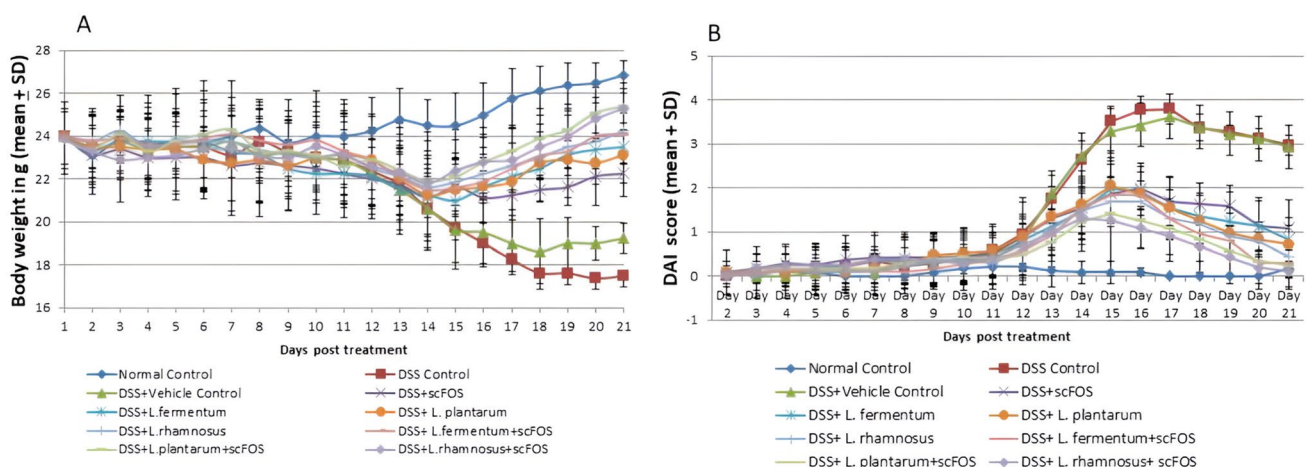


Fig. 3 Effect of treatment with lactobacilli, scFOS and combination on body weight and disease activity index. **A** Mean ± SD change in body weights of control normal mice and mice treated with vehicle

and test compounds throughout the study period. **B** Mean ± SD DAI score of control normal mice and mice treated with vehicle and test compounds throughout the study period

Measurement of TNF- α , IL-1 β , IL-17, IL-6 and IL-10 in Serum by ELISA

All the treatment groups showed significant reduction ($p < 0.05$) in inflammatory cytokines—TNF- α , IL-1 β , IL-17 and IL-6 levels in serum when compared to DSS control [2.5% DSS + Saline] and vehicle control on days 15 and 21. After the treatment with lactobacillus and scFOS, the levels of pro-inflammatory cytokines were close to that of the normal control group. The levels of pro-inflammatory cytokines (i.e. TNF- α , IL-1 β , IL-17, IL-6) in serum were attenuated, and anti-inflammatory cytokine IL-10 was upregulated in serum on both days 15 and 21 when compared to DSS control [2.5% DSS + saline].

The synbiotic test samples of *L. plantarum* and *L. fermentum* show significant decrease ($p < 0.05$) in pro-inflammatory cytokines (i.e. TNF- α , IL-1 β , IL-6) and increase in anti-inflammatory cytokine in serum when compared with respective probiotic samples whereas IL-17 was non-significant ($p > 0.05$). The combination of *L. rhamnosus* and scFOS did not exhibit any significant difference when compared to the efficacy of the stand-alone probiotic (Fig. 4).

Measurement mRNA Expression of the Cytokine in Colon Tissue by Quantitative Real-Time PCR Analysis

The assessment of localized inflammation in the colon revealed that the mice fed with lactobacilli and scFOS, either alone or in combination, displayed a significant downregulation of the pro-inflammatory markers TNF- α , IL-1 β , IL-17 and IL-6 and upregulation of anti-inflammatory IL-10 when compared to DSS control (2.5% DSS + saline). As observed in systemic biomarker analysis, the synbiotic test samples showed significant decrease in relative mRNA levels ($p < 0.05$) of TNF- α , IL-1 β , IL-6 and IL-17 and increase in relative mRNA of IL-10 when compared ($p < 0.05$) with respective probiotic samples (Fig. 5).

Histopathology

At the end of the study, colon tissues were collected for HP from each animal, fixed in 10% neutral buffered saline and subjected to histopathology. H&E staining: haematoxylin and eosin (H/E) staining of 4 micron-thick tissue sections

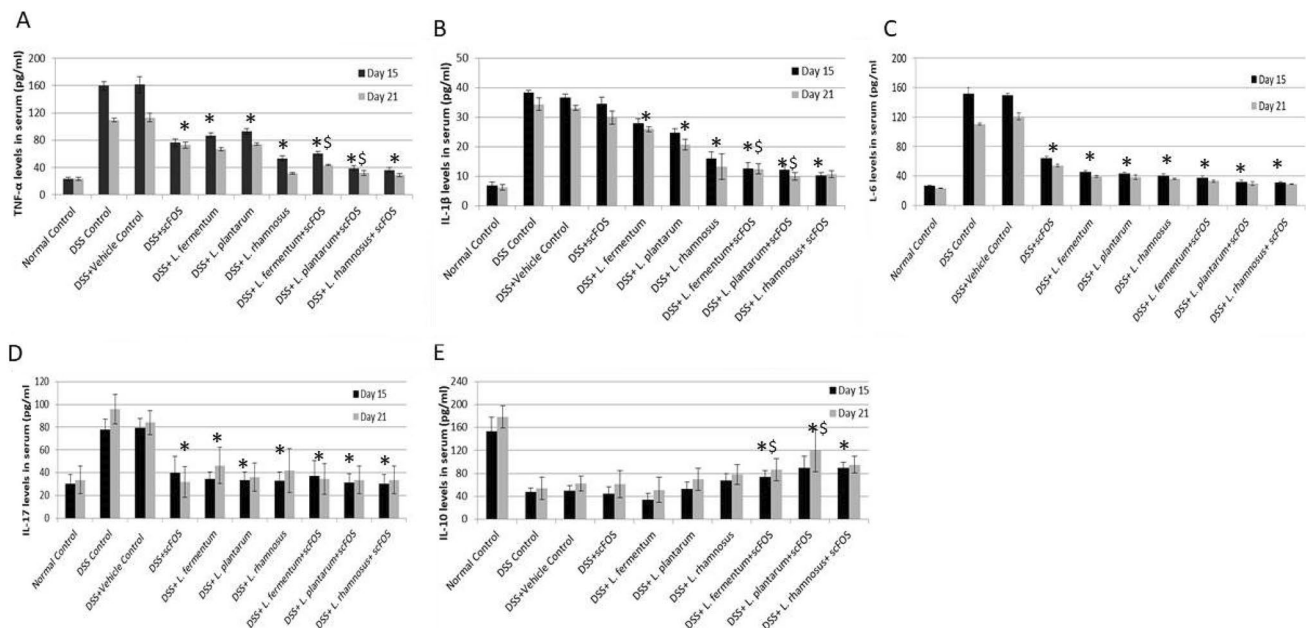


Fig. 4 Pro- and anti-inflammatory cytokine levels in serum following treatment. Blood was withdrawn from the control and treated mice on day 15 and day 21 of treatment and the levels of pro-inflammatory cytokines TNF- α (A), IL-1 β (B), IL-6 (C), IL-17 (D) and anti-inflammatory IL-10 (E) were measured. The graphs represent cytokine lev-

els in pg/ml on x-axis and the treatment on y-axis. The data is presented as mean $\pm$ SD of three replicates. *Significant difference from DSS control ($p < 0.05$); \$significantly different from respective probiotic

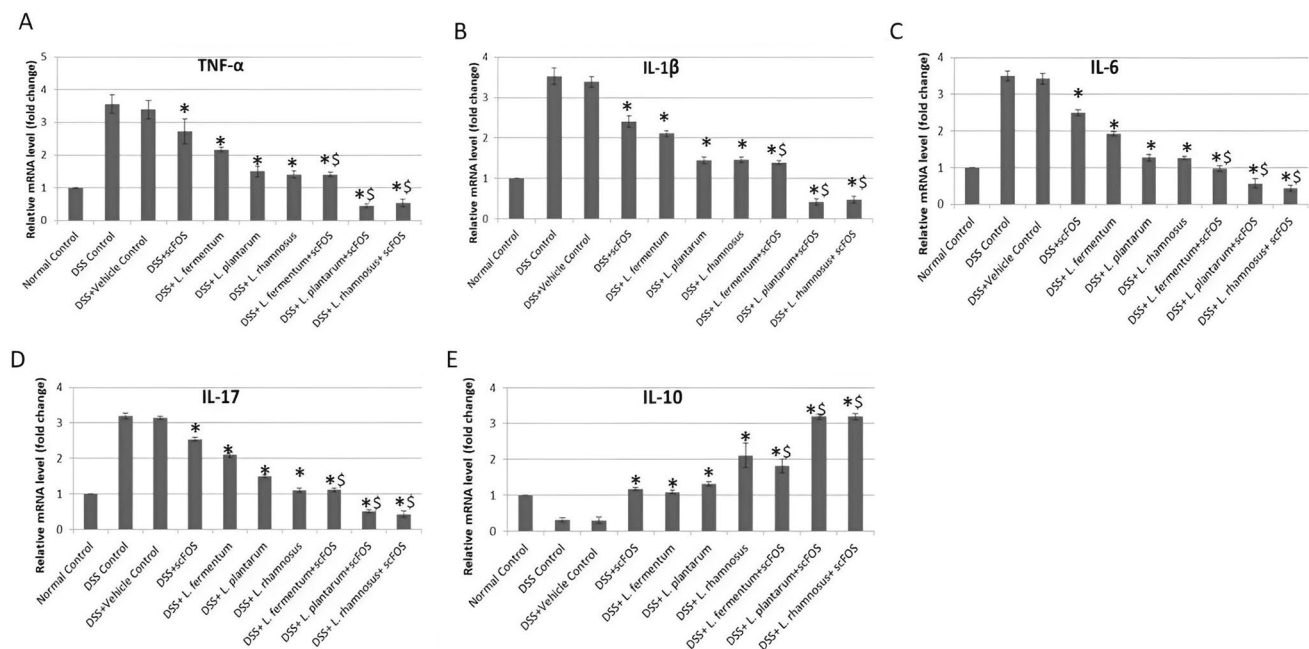


Fig. 5 mRNA expression of the pro- and anti-inflammatory cytokines in colon tissue by quantitative real-time PCR analysis. TNF- α (A), IL-1 β (B), IL-6 (C), IL-17 (D) and IL-10 (E) mRNA expression in colon tissue of normal control, DSS control, vehicle and treated

mice. The graphs represent relative mRNA expression (fold change) on x-axis and the treatment on y-axis. The data is presented as mean $\pm$ SD of three replicates. *Significant difference from DSS control ($p < 0.05$); §significant difference from respective probiotic

was performed according to the standard protocols. The total histologic score was given as epithelium (E) + infiltration (I).

Microscopic examination of colon tissues from normal control group showed normal crypts in the mucosa, sub-mucosa and muscularis mucosa. Colon tissues from disease control and vehicle control group showed mucosal erosion and loss of goblet cells, inflammation, oedema and infiltration of inflammatory cells in submucosa and muscularis mucosa when compared with normal control group animals. However, the incidence and severity of these lesions (recorded in G2 group) were reduced in treatment groups suggesting reversible nature of the change (Fig. 6).

Discussion

Ulcerative colitis is a chronic inflammatory condition that impacts the inner lining of the large intestine and rectum. It ranges from mild to severe, and in certain extreme cases, there exists predisposition to colon cancer. There is no cure for UC; the therapy involves management of symptoms through medications and surgery. In UC patients, there is dysbiosis due to abundance of bacteria that stimulates an inflammatory response in the lumen. Probiotics function to make the mucosal immune system more anti-inflammatory [9].

Studies have indicated that probiotics are more efficacious when used in combination with a prebiotic. The term “synbiotic” was coined to demonstrate synergism between the prebiotic and probiotic component. The prebiotic should be utilized by the probiotic organism that leads to metabolite production. The synbiotic is intended to enhance the in vivo survival and activity of probiotic to promote or enhance the beneficial properties [10]. The decisive factor in the development of synbiotic formulation is that the prebiotic component should selectively favour the growth and metabolite production by the probiotic counterpart. Metabolite production is very important for the maintenance of human health. Acetate, propionate and butyrate are the most widely produced SCFA that have been studied for their multifaceted health impacts. Accumulation of these SCFAs and metabolites have demonstrated beneficial effect in the maintenance of a healthy gut microbiota by hindering the adhesion and colonization of pathogens [11, 12]. In this study, the *L. fermentum* and *L. plantarum* strains could grow in specially crafted minimal media with scFOS as the sole carbohydrate source. The production of acetic acid and lactic acid was analysed in the fermented media. Both the strains displayed metabolite production in scFOS-containing media with *L. plantarum* exhibiting higher metabolite titre compared to *L. fermentum*. The growth kinetics demonstrated a better profile for *L. plantarum* compared to *L. fermentum*.

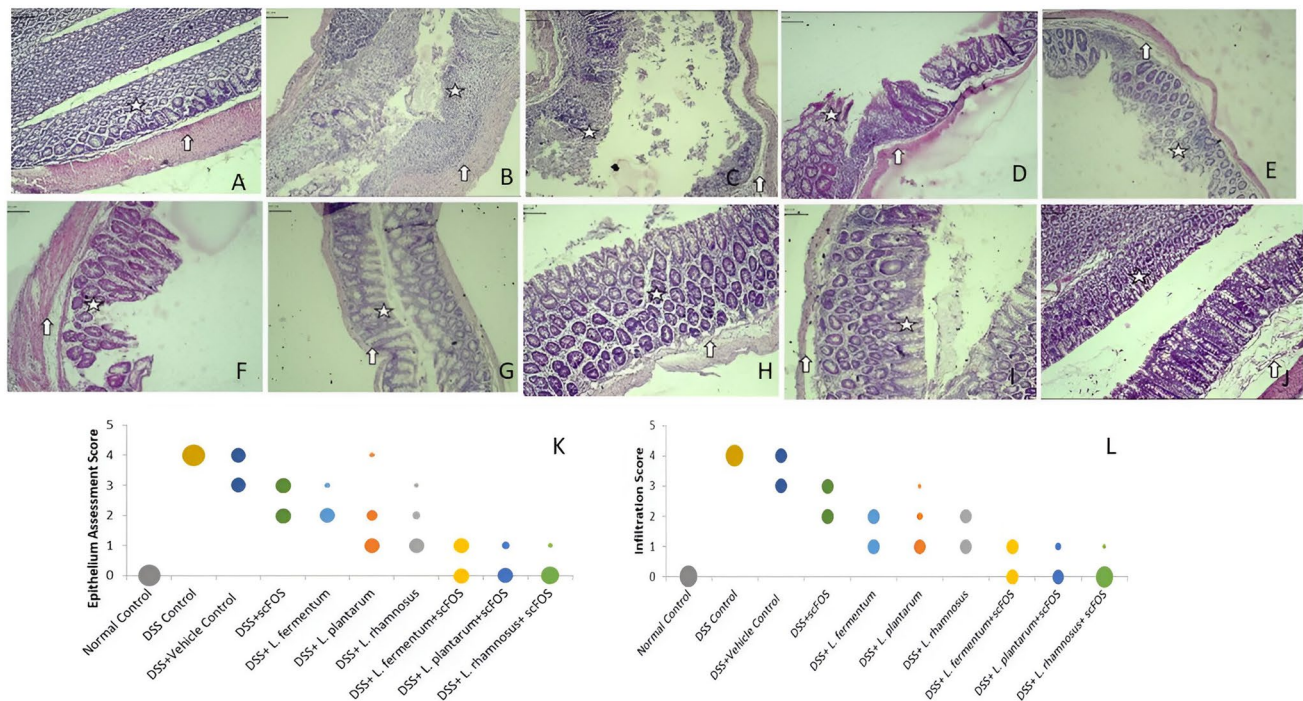


Fig. 6 Colon histopathology analysis. **A–F** Representative photomicrographs of colon histopathology following H&E staining (100×) in normal mice, DSS control mice and mice treated with vehicle, scFOS, *L. plantarum*, *L. fermentum*, *L. rhamnosus*, *L. plantarum* + scFOS, *L. fermentum* + scFOS and *L. rhamnosus* + scFOS for 21 days. The dot plots represent the epithelial assessment score or E score (**G**) and the immune cell infiltration score or I score (**H**). The

scoring is based on the following criteria: E-score: 0=No abnormality detected, 1=Loss of goblet cells, 2=Loss of goblet cells in large areas, 3=Loss of crypts, 4=Loss of crypts in large areas; I-score: 0=no infiltrate, 1=infiltrate around the crypt basis, 2=infiltrate reaching the *L. muscularis* mucosae, 3=extensive infiltration reaching the *L. muscularis* mucosae and thickening of the mucosa with abundant oedema, 4=infiltration of the *L. submucosa*

Various strains of *L. plantarum* and *L. fermentum* have been documented to utilize scFOS and produce metabolites [13, 14]. *L. rhamnosus* GG is a non-FOS fermenting strain; and hence, we do not get any appreciable growth or metabolite production in presence of scFOS [15–18]. The pro-, pre- and synbiotics are currently developed to aid restoration of the gut microbiota that will help in combating inflammation and alleviating symptoms of colitis. Several studies in mice with colitis have been performed to assess the effect of probiotics and prebiotics on the gut microbiota and disease management. Study conducted on probiotic *L. plantarum* HNU082 in colitis-induced mice demonstrated improvement in the intestinal mucosal barrier and reduction of inflammation [19]. Administration of *L. plantarum* LP3 significantly attenuated disease activity, histopathology and pro-inflammatory cytokine level in colitis-induced mice [20]. Studies have also explored the efficacy of *L. fermentum* in management of colitis in mice through improvement in body weight, disease activity index, colon length, caecal weight and histological scores [21]. In the present study, we have demonstrated the in vitro and in vivo efficacy of indigenous lactobacilli strains, *L. plantarum* and *L. fermentum*, to effectively lower inflammatory markers. The in vitro assays,

using CFS generated by growing the bacteria in a special media designed to contain scFOS as sole carbohydrate source, demonstrated significant reduction of TNF- α and nitric oxide and improvement in levels of IL-10 production in macrophage cells. Taking lead from the in vitro data, the efficacy of prebiotic, probiotics alone and in combination was conducted in mice model of DSS-induced colitis. The effect on the systemic and localized inflammatory response was studied. Significant reduction in pro-inflammatory markers TNF- α , IL-1 β , IL-17 and IL-6 and improvement in anti-inflammatory marker IL-10 were observed in serum and colon tissue samples of mice treated with the lactobacilli, scFOS and combination. There was a significant decrease in colon and serum markers—TNF- α and IL-1 β —and increase in IL-10 expression in the mice treated with scFOS and lactobacilli combination as compared to stand-alone lactobacilli. Notably, though the in vitro studies demonstrated that *L. rhamnosus* GG exhibited limited capacity to utilize scFOS, in the in vivo study, the mice administered combination of *L. rhamnosus* GG with scFOS showed enhanced efficacy compared to the probiotic-treated group in reduction of localized inflammation of colon. This effect may be due to the supportive function of other metabolites, produced

when scFOS is utilized by other resident gut bacteria, on the growth of *L. rhamnosus* GG. More conclusive evidences could be drawn from studies conducted in germ-free mice. Additionally, diverse metabolites other than lactic acid and acetic acid may also be crucial for the in vivo efficacy; to address this limitation, a holistic approach encompassing global metabolite profiling is warranted.

DSS treatment is marked by disruption of colon tissue architecture, oedema and infiltration of immune cells. The histopathological analysis of the colon showed improvement in the colon histology in the mice of all treatment groups. However, the probiotic- and synbiotic-treated mice showed a very low score indicating significant healing of colon tissue. There was a significant improvement in the epithelial morphology and reduced infiltration of immune cells in the probiotic and synbiotic group. Amongst the two, the mice administered combination of pre- and probiotic showed a better recovery pattern. Therapeutics, such as anti-TNF antibodies, that modulate inflammatory factors are used as standard therapy in IBD [22]. Due to their ability to down-regulate TNF- α and other inflammatory markers and heal colon damage, these strains have the potential to be used as therapy for management of gut inflammation.

A number of preclinical and clinical studies have documented the benefit of using prebiotics and probiotics in combination. Studies in mice models have shown the enhanced efficacy of synbiotics in alleviating gut inflammation and restoration of the microbiome [23–25]. Studies conducted on colorectal cancer models showed the ability of synbiotics to prevent the formation of pre-neoplastic lesions indicating its potential in reducing the risk of colon cancer [26]. Clinical studies, though limited, have indicated the benefits of intervention with synbiotics in management of clinical IBD. Intervention with synbiotics has been documented to improve intestinal microbiota and decrease the levels of pro-inflammatory markers with a concomitant increase in the anti-inflammatory markers [22, 27, 28]. A study on UC patients demonstrated improvement in clinical outcome on administration of a combination of *Bifidobacterium breve* plus galacto-oligosaccharide [29]. In another study, patients treated with combination of *B. longum* and prebiotic fructo-oligosaccharide and inulin exhibited decrease in the levels of inflammatory markers, CRP, TNF- α and IL-1 α [30]. Though probiotics and prebiotics have been evaluated in animal models and clinical trials, in most of the trials, the individual efficacy of the strain and the prebiotic is indeterminate. Experimental designs need to be more holistic encompassing all the ingredients used in the formulation. Clinical trials should be large and meticulously designed to evaluate the dose, duration and efficacy of individual prebiotics, probiotics and the combinations. In this study, we have evaluated the efficacy of probiotics, prebiotics and synbiotic combination. The synbiotic combination shows enhanced efficacy as

compared to individual ingredients indicating the potential of lactobacillus and scFOS combination to be used for alleviating symptoms of colitis. However, human intervention trial is warranted to ascertain the clinical efficacy of the ingredients and formulations.

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Author Contributions RP: Planning, execution, data analysis, drafting DJ: Fermentation, Sample preparation AA: Experimentation, data analysis, compilation JS: Test sample preparation and analysis AKD: Mentoring, proof reading, experimental planning

Declarations

Conflict of Interest Tata Chemicals Limited is the manufacturer of short-chain fructooligosaccharide under the brand name Fossece. But the outcome of the study is unbiased and is not influenced this fact.

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