Lactobacillus fermentum (MTCC-5898) based fermented whey renders prophylactic action against colitis by strengthening the gut barrier function and maintaining immune homeostasis

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Research Article

Keywords: Fermented whey, Probiotics, Ulcerative colitis, Gut-barrier integrity, Immunomodulation

Posted Date: August 8th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1882997/v1

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Abstract

Nutritional intervention using probiotic fermented dairy product has emerged as a promising therapeutic strategy to curb inflammatory bowel diseases. The present study was aimed to investigate the prophylactic capability of probiotic *Lactobacillus fermentum* (LF:MTCC-5898) fermented whey on impaired barrier function in DSS induced mice. Probiotic fermented whey (PFW) consumption improved the symptoms of colitis-associated with intestinal inflammation by significantly (*p* < 0.01) diminishing the percent loss in body weight, disease activity index and spleen index with improvement in colon length and weight besides hematological and histopathological score. Likewise, pre-treatment with PFW improved the barrier integrity (*p* < 0.01) in contrast to leaky condition induced by DSS administration characterized with increased permeability of FITC-dextran (451 ± 225.1) across epithelium. PFW consumption also provided the gut immune protection that occurred via significant increase (*p* < 0.05) in TLR-2 expression and stimulated T-regulatory response by producing TGF-β (*p* < 0.01), to potently suppress (*p* < 0.01) induced inflammatory response markers (TNF-α, IL-4 and C-reactive protein). Consistently, in preventive way, PFW intake significantly enhanced (*p* < 0.05) immunoglobulin (sIgA) secretion and concomitantly restored the Occludin, ZO-1 (*p* < 0.01), Claudin-1(*p* < 0.05) transcriptional expression as compared to colitis mice. Additionally, immune-fluorescence further established the presence of intact actin cytoskeleton and tight junction proteins (claudin-1, occludin and ZO-1) after PFW consumption. Thus, PFW rectified the impaired and leaky barrier junctions not only through modulation of transcriptional expression of tight junction genes but also with reduced secretion of inflammatory mediators and helped in ameliorating the colitis. Hence, probiotic fermented whey could be used as potential prophylactic functional food in the prevention of gut ailments.

1. Introduction

Whey is a liquid fraction obtained after casein precipitation during milk coagulation, identified with 6210 proteins with various minerals and vitamins [1]. In spite of such nutritional vastness, for several years, it has been considered as waste by the dairy industry. Moreover, the high biological oxygen demand of the whey effluent owing to its high organic content had also raised a concern amongst the scientific community regarding its safe disposal [2]. Furthermore, pertaining to the increased number of health issues related to protein deficiencies, majorly in developing countries, a need for supplementary source of protein have become an utmost requirement [3]. Until recently, due to high nutritional quality of whey and its products, it has emerged as an essential food ingredient and as a health enhancing nutraceutical. Moreover, whey has gained lots of popularity in terms of conferring benefits against a wide range of metabolic diseases such as cardiovascular complications, hypertension, obesity, diabetes, cancer, phenylketonuria [4]. Whey also constitutes lactose as a structural carbohydrate, providing a substrate for the growth and multiplication of lactic acid bacteria (LAB). Notably, LAB has been extensively used as a starter culture in the fermented food industry due to their proteolytic action that could increase their digestibility and hydrolyze allergenic peptides [5,6]. A potential application of fermentation includes the enhancement in the nutritional interest of the product and more valuable compounds are being produced.
such as bioactive peptides and LAB cell wall components that may interact with specific receptors in immune cells and selectively influence the immune system through different mechanism.

The dynamic dietary habits, especially ever increasing transformation from traditional to western style diet, have increased the incidence of gut-related diseases amongst the varied population [7]. IBD or inflammatory bowel disease is one such ailment which is majorly impacted by such diet practices in addition to several other environmental factors. It is a multifactorial disease consisting of ulcerative colitis and Crohn's disease, whose progression leads to loss in gut barrier function. Although the intricate and balanced system of intestinal epithelium are poised to defend against pathogenic intrusions and curtail immune response but impact of such environmental factors often results intestinal dysbiosis and immune disequilibrium, resulting in such ailment [8]. Retrospectives studies regarding the use of antibiotics in IBD treatment have emerged out to be conflicting in terms of their side effects. Besides, the re-emission of IBD symptoms, post- surgeries and treatment has further raised a need for an alternative with more effective results. Evidently, the exploitation of whey proteins for treatment of various gastro-intestinal disorders have been demonstrated lately [9]. Moreover, the nutritional value and reasonable cost of the whey have further made it a potential candidate for the food industry to utilize it in such disease prevention [10]. More recently, the positive effect of fermented whey on gut immune system have also been reported [11]. Such pieces of evidences are the basis of the current investigation involving fermented whey in the IBD prevention. As earlier in our laboratory, probiotic *Lactobacillus fermentum* (LF:MTCC-5898), isolated from infant feces, had shown promising immunomodulatory attributes and anti-oxidative capacity in aged mice [12]. In addition, variable doses of *L. fermentum* were also found safe and nontoxic to weaning mice after four weeks of orogastric challenge [13]. Furthermore, *in vitro* studies have shown the ability of the *L. fermentum* (LF: MTCC-5898) to ameliorate the *E. coli* induced barrier dysfunction and maintain immune homeostasis [14,15]. Therefore, further investigation is required to unveil the process of gut immune homeostasis imparted by probiotic fermented whey in a diseased milieu. Hence, the present study was aimed to determine the effect of dietary supplementation with probiotic *L. fermentum* (LF: MTCC-5898) based fermented whey on gut barrier integrity associated immunomodulation in DSS-induced ulcerative colitis mouse model.

### 2. Material And Methods

#### 2.1 Probiotic culture maintenance

Probiotic *L. fermentum* MTCC-5898 used under the present investigation was isolated from infant feces. For *in-vivo* experiments, culture was stored at -80°C in de Man-Rogosa-Sharpe (MRS) broth (Hi-media, Mumbai, Maharashtra, India) supplemented with 20% (v/v) glycerol. Before the start of the experiment, culture was propagated twice in MRS broth for 18 h at 37°C.

#### 2.2 Preparation of probiotic fermented whey for treatment groups

To prepare sweet whey, milk was procured from the experimental dairy of ICAR-National Dairy Research Institute (NDRI), Karnal, India. Initially, milk was added with enzyme-rennet that coagulated the casein
from which whey was drained out [16]. Prepared sweet whey was supplemented with 4% whey protein concentrate (Davisco foods International, Inc., Minnesota, USA) and pasteurized before fermentation. Fermentation was performed in sterile bottles with the inoculation of 2% (v/v) activated culture and incubated for 12 h at 37°C until reached the stationary phase. Bacterial count in fermented whey was checked through plate count on MRS agar plates exhibited 1×10^9 colony-forming units (CFU/mL) in 12 h. After incubation, fermented whey was centrifuged at 7000 rpm for 15 min at 4°C to collect the supernatant. The purity of culture was confirmed through Gram staining every time before the start of the experiment.

2.3 In-vivo experimental design and DSS-induced colitis model

2.3.1 Animals

Swiss albino male weaning mice aged 4 weeks weighing about 16-19 g were obtained from a small animal house of ICAR-National Dairy Research Institute Karnal, Haryana, India. All the experiments were performed with the approval of Institutional Animal Ethics Committee (IAEC) approval (Registration no. 1705/GO/RE/SL/13/CPCSEA/ 41-IACE-18-60 dated 31/05/2019). Animals were housed in polycarbonate cages at a temperature (24±2°C) and relative humidity (55±10ºC) with 12 h light/dark cycles. The mice were adapted for one week before the start of the experiment and were fed *ad libitum* on a semi-synthetic basal diet (BD) (11·5% starch, 70% bengal gram (black chickpea) our, 6·5% soyabean oil, 2·1% mineral mixture, 1% vitamin mixture and 8.9% cellulose).

2.3.2 Colitis induction and animal grouping

Ulcerative colitis in mice was generated by adding 5% (w/v) dextran sodium sulfate (DSS, MW-500, Sisco Laboratories, Pvt. Ltd, Mumbai, Maharashtra, India) in their drinking water *ad libitum* for 6 days. During the experimental study, all animals were randomly assigned into five different feeding groups with each group containing nine animals. Group I (Control) mice were fed with dough prepared from the basal diet (5g/animal/day) with *ad libitum* supply of drinking water. Group II (DSS) mice were fed with the basal diet but drinking water was replaced with 5% DSS from 23rd day onwards for six days to induce colitis. Group III (W+DSS) mice were fed with basal diet supplemented with sweet whey (2mL/animal/day) during preparation of dough instead of water to obtain consistency and administered with DSS after 23rd day onwards as in previous group. Group IV (PFW+DSS) mice were fed with dough of basal diet prepared with probiotic fermented sweet whey having 2.0×10^9 cfu/animal/day instead of water to obtain consistency and subsequently challenged with 5% DSS as in case DSS induced colitis group. Group V (PFWS+DSS) mice were fed as in group IV but, instead of probiotic fermented sweet whey, supernatant of this preparation was used which was devoid of bacteria for the preparation of dough and subsequently challenged with DSS. The general conditions and health of the animals were monitored by routine measurement of body weight and periodic observation of clinical symptoms. At the end of the respective study period, animals were euthanized by diethyl ether overdose and then blood, spleen, liver, kidney, intestine of each animal were collected to assess various barrier and immunological parameters.
2.4 Collection of sample

At the end of the experimental period on the 29\textsuperscript{th} day, all the animals were sacrificed by euthanization. Blood was collected by cardiac puncture from the heart and used for hematological assessment. The intestinal sections were flushed with 2.5 mL PBS (pH 7.4) followed by centrifugation at 2000 g for 10 min at 4\textdegree C [17]. The intestinal fluid was collected and stored at -80\textdegree C for enzyme-linked immunosorbent assay (ELISA). Intestinal tissue was also stored in RNA later (Sigma-Aldrich, St Louis, Missouri, USA) until RT-qPCR analysis was carried out. Intestine tissue sections stored at -80\textdegree C were also used for MPO analysis. Tissue sections fixed in 10\% formalin and embedded in paraffin, 3 \textmu m thick sections of the tissues were prepared with Senior Rotary Micrometre (Radical, RMT-30, Ambala, Haryana, India) and used for histological and immuno-fluorescence assessment.

2.5 Body weight and clinical assessment of parameters

Body weight of all the test animals was checked weekly and compared to the average weight in respective groups. The disease activity index (DAI) was calculated as the sum of the score for weight loss, stool consistency and blood in feces following criteria [18] of the clinical scoring system as shown in Table 1. At the end of the feeding trial, the test animals were euthanized and different organs (liver, kidney, spleen) were dissected out for the organ indices determination by dividing them with the respective body weight of the animals. Furthermore, analysis of blood was done by using automated hematological analyser (MS4Se-Melet Schloesing Lab, New Delhi, India) for routine parameters such as hemoglobin (Hb), total red blood cell (RBC), white blood cell (WBC), lymphocyte, neutrophil, mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC).

2.6 Intestinal morphology

The fixed slides were stained with hematoxylin and eosin stain for histological analysis and were evaluated under the light microscope (40x magnification). Histopathological damage scoring was performed by assigning scores depending upon the destruction of the crypt structure (0-4: basal one-third damage, basal two-third damage, only surface epithelium intact, entire crypt, and entire epithelium lost), the depth of the lesions (0-3: none, mucosal, submucosal, transmural) and the degree of inflammatory cell infiltration (0-3: none, slight, moderate, severe) followed by their summation [19].

2.7 Myeloperoxidase (MPO) activity

Neutrophil infiltration in colon was assessed indirectly by measuring MPO activity. All procedure was performed for MPO activity according to manufacturer instructions [20]. Briefly, the colon tissue (50 mg/mL) suspended in potassium phosphate buffer (50 mM, pH 6.0) having 0.5\% HTAB was sonicated for 10 sec and freeze thawed repeatedly for 3 cycles. Later, tissue suspension was centrifuged at 12,500 rpm for 5 min at 4\textdegree C. The MPO activity in the supernatant was measured at 460 nm (UV-visible double beam spectrophotometer) at every 30 sec interval for 5 min after incubating 0.5\% of o-dianisidine dihydrochloride and 1\% H\textsubscript{2}O\textsubscript{2} as substrate. One unit of enzyme is defined as the amount which converted
1 μM of the substrate into product per mg of tissue per min considering the extinction coefficient of o-dianisidine to be $1.13 \times 10^4$/M/cm at 28°C.

### 2.8. Cytokine response in intestinal fluid

Cytokines secretions such as IL-4 (eBiosciences, San Diego, California, USA), TNF-α (Biolegend Inc., San Diego, California, USA), TGF-β (Invitrogen, Thermo fisher scientific, Vienna, Austria) were evaluated by enzyme linked immunosorbant assay in intestinal fluid collected from the intestine as per the manufacturer protocol [17]. In brief, 96 well immune plates (Hi-media) were coated with 100 μL/well capture antibody and incubated overnight at 4°C. The next day after PBS washing, 200 μL of blocking buffer was added to each well to avoid non-specific binding. Afterward, undiluted intestinal fluid samples were added in experimental wells followed by the addition of 100 μL detection antibody of respective cytokines. Later, 100 μL/well HRP-conjugated avidin was added as a detection enzyme. Finally, TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03% H₂O₂) was added to develop color and the reaction was stopped by 2 N H₂SO₄. Plates were read at 450 nm automated ELISA plate reader (BioTek Instruments, Winooski, Vermont, USA). Moreover, C-reactive protein (CRP) was also estimated in intestinal fluid. Pre-coated anti-CRP (Boster Immunoleader, Pleasanton, California, USA) microplate strips were added with 100 μL of the undiluted sample of intestinal fluid and incubated for 120 min at room temperature (RT) then added with biotinylated anti-mouse CRP antibody for 90 min at RT followed by the addition of 100 μL of Avidin-Biotin-Peroxidase complex. Lastly, 90 μL of colour developing reagent was added to each well and the reaction was stopped by using the stop solution. At the end, plates were read at 450 nm by using a microplate reader (BioTek Instruments, Winooski, Vermont, USA). Results were expressed pg/mL.

### 2.9 Estimation of secretory IgA

Total secretory IgA was estimated by ELISA in intestinal fluid. In detail, 96 well plates (Hi-media) were coated with 100 μL of coating antibody in 50 mM carbonate-bicarbonate buffer and incubated at 4°C overnight. Afterward, 200 μL with blocking solution (1% bovine serum albumin in PBS) was added at room temperature for 1 h to block free sites. Then 100 μL of diluted samples (1:1000 times) were added to respective wells followed by the addition of 100 μL of horseradish peroxidase (HRP) anti-mouse IgA antibodies. Finally, 100 μL TMB (3,3,5,5-tetramethyl diaminebenzidine containing 0.03 % H₂O₂), was added to the plate and allowed to develop color at room temperature and the reaction was stopped with 50 μL of 2 N H₂SO₄. Plates were read at 450 nm on a microplate reader (BioTek Instruments, Winooski, Vermont, USA). Results are expressed in μg/mL.

### 2.10 Transcriptional expression of tight junctional and immune related genes by RT-PCR

Intestinal tissue was placed in RNA later stabilizing reagent (Sigma-Aldrich). Total mRNA was extracted from tissue following trizol method as described in manufacturer’s protocol and further used for relative quantification of genes associated with key tight junctional and immune genes. RNA integrity was
determined by gel electrophoresis in 1.5% agarose gel. The purity of RNA was assessed by ratio of absorbance at 260 and 280 nm using micro-volume spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). RNA purity was within range of 2.0-2.1. Briefly, RNA (1μg) was converted to cDNA using reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was stored in -20°C until further use. Quantitative real time PCR analysis was done to determine relative amplification of target gene such as Occludin, Claudin-1, Zonula-occludin-1 (ZO-1), human β-defensin-2 (hBD-2), TLR-2 and TLR-4 in different treatment groups by using ABI PRISM 7500 thermocycler system (Applied Biosystems, California, USA). In real time PCR plate each sample run in duplicate, total reaction volume for each reaction was 10 µL containing 1 µL of test sample, 5 µL of syber green (Thermo scientific, USA), 0.5 µL each primer, 3 µL nuclease free water. Sequence of specific primer pairs are shown in Table 2. Amplification cycle consists of initial denaturation for 5 min at 94°C, 35 cycles of denaturation (94°C for 30 sec), annealing (60°C for 30 sec) and extension (60°C for 45 sec) and final extension cycle at 60°C for 5 min. After transcription, transcript level fold change between different groups were assessed from threshold (C_t) values using GAPDH as control gene [21].

2.11 Measurement of intestinal permeability

Intestinal epithelial permeability was determined through FITC-dextran (4 kDa; Sigma-Aldrich) in blood after it was orally administered as described previously [22]. After overnight fasting, each mouse received FITC-dextran 60 mg/100g of body weight. Blood sample was collected 4 h later, was first centrifuged 2000 g for 10 min at 4°C and serum was collected. Serum was diluted by a ratio of 1:10 with PBS and then 100 µL of diluted serum was added in black 96 well microtiter plate (SPL Life Sciences, Korea). The concentration of FITC-dextran was determined by spectrophotofluorometry based Multimode Plate Reader (TECAN, infinite M200 Pro, Austria) using excitation wavelength of 485 nm and emission wavelength of 535 nm using non-treated serially diluted serum (1:10 v/v with PBS) as standard.

2.12 Immunofluorescence labelling of intestinal tissue TJ proteins and cytoskeleton component

To investigate the effect of PFW on tight junction proteins, they were localized by immune-histochemical staining [23]. The histological sections were processed for immune staining using fluorescent antibodies against claudin-1 (0.2 mg/mL; 1:200 dilution, Thermo fisher Scientific, Massachusetts, USA), occludin (0.25 mg/mL; 1:80 dilution) and ZO-1 (0.25 mg/mL; 1:80 dilution). The sections were deparaffinized and rehydrated. After rehydration slides were rinsed with PBS (pH 7.4) thrice and then unmasked the antigen by boiling in 10 mM boiling citrate buffer (pH 6.0) for 15-20 min followed by permeabilisation with 0.05% Triton X-100. To reduce nonspecific binding, sections were blocked with 2% bovine serum albumin (BSA) for 1 h followed by incubation with primary antibodies against TJ proteins for 2 h. Subsequently, cells were washed with PBS and incubated with FITC labelled anti-rabbit goat IgG antibody (1.5 mg mL; 1:200 dilution, Thermo fisher Scientific, Massachusetts, USA) for 1 h. Finally, sections were abundantly washed five times with PBS and tight junction framework were visualized under Olympus optics BX60 microscope (Olympus, Tokyo, Japan). Similar to above experiment, cytoskeleton actin filaments were visualized with FITC-labelled phalloidin which has specificity for actin. Sections were deparaffinised and rehydrated
followed by boiling in 10 mM citrate buffer (pH 6.0) and after that stained with FITC conjugated phallodin (0.1 mg/mL, 1:100, Sigma-Aldrich) for 2 h. To remove nonspecific binding, profuse washing was done with PBS five times. At last immune-histochemical assessment was performed under fluorescent microscope (Olympus optics BX60 microscope, Olympus, Tokyo, Japan).

2.13 Enumeration of total lactic acid bacteria (LAB) and *E. coli* count in feces

In order to estimate fecal lactobacilli and *E. coli* count, feces were collected after completion of 28 days feeding before the animals were sacrificed. Freshly collected fecal matter (100 mg) was homogenized in sterile PBS (pH 7.4) than diluted serially (10^5) followed by plating. For the enumeration of total lactobacilli count, plating was done on MRS agar and to check *E. coli* count, plating done on eosin methylene blue agar (EMB). Plates were incubated at 37°C for 24-48 h for morphological assessment of colonies and results were expressed as log_{10} CFU/g weight of feces.

3. Statistical Analysis

Data was analysed by GraphPad Prism (Version 5.01, San Diego, CA, USA) software. Results were expressed mean ± SEM (standard errors of the mean) and mean ± SD (standard deviation). Differences between the mean were tested for the statistical significance (p < 0.05) using one-way analysis of variance (ANOVA) with posthoc Tukey’s and two-way ANOVA with posthoc Bonferroni posttests multiple comparison test were performed to evaluate the significant differences between the groups.

4. Results

4.1 Improvement in health score during induction of colitis with DSS

This study first investigated the potential preventive effect of probiotic fermented whey in mice with DSS-induced colitis. PFW, PFWS and whey-colitis groups ameliorated the disease severity in animals though significant decrease (p<0.01) in loss of percent body weight as compared to the DSS-colitis animals (Fig. 2A). The DSS and W+DSS fed-groups displayed significantly increased (p<0.01) disease activity index of 8.1±0.98 and 5.3±0.80 respectively after the sixth day of colitis as compared to animals groups where PFW and PFWS fed test groups had DAI of 2.3±0.55 and 3.0±0.51 respectively (Fig. 2B). Additionally, DSS typically caused an increase in the spleen index whereas PFW fed animals showed improvement with a significant decrease (p<0.05) in the spleen index (Fig. 2C). In contrast, the results of liver and kidney indices in animals of different treatment groups did not show any statistical changes in comparison to the DSS-colitis group as shown in Fig. 2D&E. Moreover, DSS administered animals representing the inflammatory condition with the higher number of payer patches in the intestine which were reduced remarkably near to healthy control animals after PFW (p<0.01) and PFWS or whey(p<0.05) consumption respectively even after colitis induction in them (Fig. 2F). Furthermore, consumption of probiotic fermented whey improved the number of RBC, hemoglobin content, MCHC and neutrophils (p<0.01) during the induction of colitis with DSS (Table 3).
4.2 Maintenance of colon health and mitigated the colonic histopathological changes

Chronic colitis was characterized by a marked reduction in colon length and colon weight on induction of colitis. Colon shortening was used as a marker for inflammation and indirectly indicated the severity of the disease. DSS induced intestinal inflammation significantly (p<0.01) reduced the weight and length of the colon. Such change was significantly improved (p<0.01) by PFW and its PFWS administration through the maintenance of colon length similar to healthy control. Similarly, reduction in colon weight was also significantly improved in PFW (p<0.01) and its PFWS (p<0.05) fed animals in contrast to the DSS-colitis group (Fig. 3A,B). Moreover, the results in improvement of severity of chronic inflammation and ulceration were determined through hematoxylin and eosin (H&E) staining (Fig. 3E). Colons of healthy control group mice exhibited normal integrated architecture and intact morphology of crypt with a low histological score. The DSS-colitis and whey-colitis group exhibited significantly higher (p<0.01) histological score with severe epithelial damage, crypt loss and abundant inflammatory cell infiltration mainly in mucosa and sub-mucosa in contrast to healthy control animals. Conversely, PFW fed animals reduced the extent of colon injury and inflammatory cells showing lower histologic score (p<0.01) while the PFWS-colitis fed group did not display a significant difference in histopathological score. The histopathological score also related with the results of hematoxylin and eosin staining (Fig. 3C).

4.3 Modulation in the secretion of inflammatory mediators in colonic tissue

The level of MPO reflects the number of neutrophils [24] as shown previously through histopathological examination. MPO activity of DSS colitic animals was significantly increased (p<0.01) as compared to healthy control group animals indicated in Fig. 3D. The pre-feeding of PFW, PFWS and whey in colitis induced animals evidently reduced (p<0.01) the MPO activity in contrast to DSS-colitis group animals. The immunomodulatory effects of PFW and PFWS in DSS induced colitic mice was analyzed through ELISA at the protein level. The production of proinflammatory cytokines such as TNF-α, IL-4 along with C-reactive protein (CRP) was significantly higher (p<0.01) in DSS-induced colitic mice in comparison with control animals (Fig. 4A-C). Pre-feeding of PFW (p<0.01) before colitis induction with DSS prevented the inflammatory response by significantly decreasing the secretions of these inflammatory markers TNF-α, IL-4 and CRP efficiently. Whereas, PFWS group animals displayed a significant decrease (p<0.01) in TNF-α only while an insignificant change in other inflammatory markers (IL-4 and CRP) was observed as compared to the DSS-colitis group animals. In contrast to this, PFW fed animals showed a significant upsurge (p<0.01) in immunoregulatory TGF-β as compared to other experimental test groups (Fig. 4D). All of these results showed that the PFW exerts an inflammation-regulating effect via influencing the secretion of specific regulatory cytokine (TGF-β). Likewise, the impact of probiotic fermented whey on the level of secretory IgA was analyzed in intestinal fluid. As shown in Fig. 4E level of sIgA increased tremendously (p<0.05) on the administration of PFW in colitis induced animals exhibiting a defensive response.

4.4 Decreased the intestinal permeability by improved barrier functions
The potential mechanism behind reinforcement is expected to involve the key pattern recognition receptors TLR-2 and TLR-4 signaling pathway. TLR-4 expression was significantly increased by 3.1 ± 0.9 fold in colitis induced animals as compared to normal control. Conversely, PF exhibited a significant increase in the expression of TLR-2 by 4.97±2.44 fold than control group (Fig. 5A,B). The transcription level of occludin, claudin, ZO-1, hBD-2 in mouse colon tissue was assessed by real-time quantitative PCR (qPCR) (Fig. 5C-F). The expression level of different tight junctional genes occludin, claudin-1, ZO-1 were diminished significantly by 0.81 ±0.29, 0.74±0.59, 0.70±0.64 fold respectively after DSS administration in animals. Contrarily, PFW fed animals significantly increased the expression level of occludin, claudin, ZO-1 by 3.14 ±1.82, 2.54±1.56, 2.36±1.27 fold respectively. On the other hand, the key antimicrobial component of a secretory barrier function i.e. hBD-2 expression level related to host gut mucosal immunity was decreased significantly by 25.10±6.52, 35.51±7.80 folds on PFW and its PFWS supplementation respectively as compared to DSS colitic group. Protective effects were further characterized by quantifying the leakage of FITC-dextran in the serum of mice. The results showed that the diffusion of FITC-dextran through the epithelium after DSS treatment was increased (548±451.1 ng/mL) in the intestine of mice. However, pre-feeding with PFW significantly reduced (71.0±67.5 ng/mL) the permeability of FITC-dextran across the epithelium similar to healthy control. No significant change was observed in PFWS-colitis and whey-colitis groups. (Fig. 5G). No doubt regulation of tight junction proteins is a vital component of epithelial barrier repair after injury. The immuno-staining of junctional protein claudin-1, occludin, ZO-1 and cytoskeleton actin filament in intestinal sections also established intact epithelial barriers on consumption of PFW and PFWS by appearance of regular and intense green fluorescence showing their preserved and localized distribution than DSS induced colitis (Fig. 5H).

4.5 Enhanced the fecal lactobacilli count

Effect of probiotic and whey components on the fecal bacterial count in MRS and EMB agar as shown in Fig. 6A,B. DSS colitis was associated with gross alteration in fecal flora. DSS administration decreased the lactobacilli count and increase the E. coli count in feces. In contrast to this, the inclusion of PFW and its PFWS significantly increased the lactobacilli (p<0.05) and decreased the E. coli (p<0.05) count in feces after 28 days of experimental feeding. While whey-colitis group revealed insignificant change as compared to the DSS-colitis group.

5. Discussion

Ulcerative colitis (UC) is a chronic relapsing inflammatory disorder of the gastrointestinal tract with high prevalence in developed countries [25]. Currently, classical therapies are available for the prevention and treatment of disease are not very effective and causes harmful side effects as well. Hence, as a natural health remedy, researchers are currently focusing on the dietary interventions that are based on the dairy products containing probiotic with an ability to minimize the risk of pathogen associated inflammatory disorders. Whey contains a multitude of proteins that possess immunobiological properties and is widely used in the food industry as a functional and nutritional ingredient. Additionally, it also contains essential bioactive components such as lactoperoxidase, lactoferrin, α-lactalbumin, epidermal growth factor (EGF)
and transforming growth factor (TGF) that have numerous beneficial effects on human health [26, 27]. Foods fermented by specific bacterial strains containing bioactive compounds that have been produced during the fermentation can lead to various beneficial properties [28]. Previous studies had indicated the protective role of whey on inflammatory bowel disorders such as ulcerative colitis in the animal model. Based on this, the current study has been carried out by using colitis murine model to gain insight into pre-treatment of whey containing probiotic in strengthening of impaired intestinal epithelial barrier integrity, thus making it a potential choice for the management of IBD. To establish an acute model of UC, mice were administered with 5% DSS for 6 days consecutively. DSS triggered a strong colonic inflammation accompanied by diarrhea, rectal bleeding exhibiting body weight loss and higher DAI score. The present study found that pre-feeding of PFW attenuated diarrhea and rectal bleeding with a significant reduction in percent body weight and clinical DAI score as supported by a previous study in which Kefir-treatment significantly reduced the clinical DAI in DSS-induced colitis rats [29]. On the other hand, no change was seen in the liver and kidney index except for the spleen index, which exhibited a significant increase in DSS treated colitis group than PFW group which normalized the increased spleen index similar to normal control. The spleen is a secondary immunological organ and studies have demonstrated the increase in the weight of spleen due to the hyperactivity of spleen upon immune activation during the infection [30]. Moreover, the colon length and colon weight reflected the health of the intestine, which in DSS colitic mice were significantly shortened and reduced respectively as compared PFW and PFWS groups. Represents that PFW significantly prevented the DSS-induced shortening in colon length and decrease in colon weight showing a healthy colonic environment. Substantial evidence showed that the infiltration of inflammatory cells such as neutrophil and macrophages is a hallmark of disease pathology in IBD [31]. Concerning, histopathological findings, the administration of PFW in colitis induced mice strongly restored mucosal integrity, effectively diminished the tissue destruction and neutrophil infiltration with a lesser histological score. These clinical findings are in consonance with Assis et al., 2016, wherein significant improvement occur in intestinal inflammation after treatment with goat milk and milk derived probiotic yogurt in the experimental colitis model, as evidenced through the reduction in macroscopic damage, inflammatory infiltration, oxidative stress as well as preservation in cytoarchitecture of the colon [32]. MPO is the biomarker of gut acute inflammation that is abundantly expressed in neutrophils. The present findings displayed a significantly enhanced MPO activity in DSS-colitis which has been used as histopathological marker and related directly with intestinal tissue injury. In contrast, PFW and its PFWS consumption displayed statistically reduced MPO levels, demonstrating its anti-inflammatory effects. Similarly, a study recently reported that whey treatment before colitis induction reduces the MPO activity and improves the clinical signs of the disorder by maintaining colon health [33]. Cumulatively, these observations confirms the beneficial role of probiotic fermented whey in colitis correlated to a decrease in local inflammation and preventing intestinal disturbances.

The impaired intestinal structure was also associated with increased immune activation and an exaggerated inflammatory response [34, 35]. Blood is an indicator of anemia and different parameters such as hemoglobin, number of RBC, MCHC showed much lower values while higher neutrophil are found in DSS-induced animals. Moreover, the animals treated with PFW and its PFWS had in general more
normal blood parameters (Hb, RBC, neutrophil, and MCHC) and presenting healthy general health condition. These results are in line with prior observations in which lactic acid bacteria treated diets showed improved hematological blood parameters (Hb, RBC, WBC, neutrophil) and potentially improving the immunity levels [36]. The innate immune system is the first line to recognize microbe or endogenous molecule via pathogen associated molecular patterns or damage associated molecular patterns by host pattern recognition receptors [37]. TLRs are a major component of innate immunity and plays an important role in intestinal homeostasis. A previous study reported that TLR-4 polymorphism are associated with ulcerative colitis and crohn's disease [38]. Here, under present investigation the increase in TLR-4 mRNA expression mediated by DSS administration which is related to the severity of the infection and able to trigger the inflammatory response in immune cells. However, PFW containing components promoted recovery of DSS induced colonic inflammation and this effect was potentially mediated, at least partially through TLR-2 receptor. It has also been suggested that persistence of inflammation could be prevented by enhancing epithelial resistance by TLR-2 activation and suppressing TLR-4 expression with the help of probiotic bacteria and fermented biogenic components [39, 40]. These results are also in line with the studies of Grabig et al., 2006 who revealed that probiotic E. coli Nissle 1917 modulate T-cell expansion and cytokine secretion by regulating TLR-2 and TLR-4 signaling pathway that ameliorate experimental colitis [41]. Furthermore, the current study also recorded, the elevated expression level of antimicrobial peptide (β-defensin-2) in DSS-colitis group while a significant decrease was observed in PFW and its PFWS-colitis groups. In ulcerative colitis there is an overexpression of hBD-2 mRNA which was correlated with increased expression in inflammatory cytokine TNF-α [42]. Overproduction of pro-inflammatory cytokine is a hallmark of colon damage in the development of ulcerative colitis [43]. Therefore, under present analysis, prophylactic administration of PFW and its PFWS alleviated acute colitis by significantly suppressing the higher production of proinflammatory cytokine IL-4 as well as CRP (acute phase protein) level while TNF-α showed significant decrease in only PFW fed animals. Increased TNF-α induces the CRP secretion as both regulate each other in a feedback manner [44]. Kefir treatment prevents the pro-inflammatory cytokines surge through decreasing TNF-α level in colon mucosa of DSS-induced colitis mice [29]. TGF-β is an important regulatory cytokine that trigger the differentiation of Tregs which prevents colonic inflammation and epithelial restitution during experimental colitis [45]. In concern to this, the present study indicated significantly enhanced production of regulatory/anti-inflammatory TGF-β in PFW fed animals. Similar to existing results, feeding of Lactococcus lactis fermented milk demonstrated a considerable reduction in inflammatory cytokines levels with elevated regulatory TGF-β production against collagen induced arthritis [46]. Moreover, Thomas et al., 2012 found that soluble factors released from L. reuteri inhibited the proinflammatory cytokine production and signaling immune cells [48]. Likewise, whey product has demonstrated several anti-inflammatory effects through decreased inflammatory cytokine secretion in rodent model after exposure to LPS [48]. Taken altogether, the results confirmed that fermented whey components and probiotic synergistically influences the immunological outcome by regulating the TLR-signaling which is involved in the immune activation and support the notion that innate immune signaling is required for the initiation of adaptive immune response and both are functionally interlinked.
TGF-β signaling seems to play a central role in the induction of IgA that helps in the maintenance of mucosal homeostasis. In the present study, PFW consumption increased the IgA level in the intestinal fluid which influences the gut barrier. Thus present results are in accordance with Sakai et al. 2014 who reported the similar observation where dietary intake of probiotic *L. gasseri* SBT2055 showed TLR-2 and TGF-β signals which were critical for the production of IgA and IgA (+) cell population [49]. Moreover, the intestinal microbiota is also the most important and diversity of which is associated with the instigation and aggravation of the disease [50]. Current analysis exhibited the increased fecal *E. coli* count in diseased condition whereas the PFW/PFWS fed animals showed significant decrease in the *E. coli* count. These findings are consistent with previous findings where oral administration of cheese whey in wistar rats increases the intestinal lactobacilli count leading to probiotic therapeutic effect under chronic inflammation of DSS induced colitis [51]. Studies have suggested that dairy proteins protected the probiotic bacteria via the process called coacervation. These dairy proteins pack the microorganism inside, thus forming a kind of barrier that protect the microorganisms inside from adverse environmental conditions and maintain intestinal barrier function [52, 53].

Fermented milk products such as yogurt and cheese are expanding as a food in many countries providing health promoting effects. Several studies reported that at the level of intestine, the interaction between proinflammatory cytokine and mucosal immune system can augment the disruption in tight junctional protein that disturb the intestinal homeostasis [54]. Pathogen induced infection lead to the impairment in barrier function could be due to the release of toxins and internalization of tight junctional protein [55]. Disruption in the integrity of barrier occurs in several diseases such as inflammatory bowel diseases [56]. In consonance with these findings, the present study revealed that PFW treated group showed the significant increase in the expressions of junctional genes such as occludin, claudin-1 and ZO-1 in contrast to DSS-colitic group. These findings are supported by Corridoni et al., 2012 showed that metabolites secreted by *Bifidobacterium infantis* Y1 are one of the component of probiotic product VSL#3 which leads to the increase in expression of ZO-1 and occludin leading to the increase in transepithelial resistance [57]. Additionally, pre-treatment of probiotic stimulated ZO-1 and occludin by modulating TLR-2 expression in turn restricting pathogen invasion and thus enhancing barrier function [58]. Moreover, the existing data suggest a correlation between tight junctional expression and gut permeability which could offer new insights in understanding the mechanism behind reinforcement of the mucosal barrier. Paracellular permeability is regulated by the tight junction network between epithelial cells. The findings of the current study regarding tight junctional permeability shows the significant decrease in transport of FITC-dextran level in serum of PFW fed animals which was enhanced under DSS-injury. Similarly, Rokana et al., 2016 observed a remarkable decrease in epithelial permeability in probiotic fermented milk supplemented mice through modulation of key regulatory receptors against *Salmonella* induced infection [59]. These outcomes were consistent with immunofluorescence data showing improved protein expression of cytoskeletal actin and tight junctional proteins claudin-1, occludin and ZO-1 with well-preserved intestinal epithelial structure in PFW-treated colitis induced mice. In consonance with present study, Zhao et al., 2019 revealed that bovine lactoferrin significantly increased the production of tight junctional proteins claudin-1, occludin in two cell models (Caco-2 and HIECs) [60].
Thereby indicating the regulation of inflammation condition can restore the expression of tight junctional protein or vice-versa. To our knowledge, the present study is the first to determine the effect of dietary supplementation of *L. fermentum* (LF: 5898) fermented whey on the prevention of DSS-induced ulcerative colitis and amelioration of the intestinal barrier dysfunction through providing immune-enhancing effects.

**Conclusion**

The present study revealed that potential probiotic *L. fermentum* fermented whey intervention prevents DSS-induced impaired barrier function by modulating the expression of host tight junctional and immune genes in the colitis model. Pre-feeding of PFW recovers from the inflammatory conditions by mitigating clinical disease activity, hematological, histopathological score and MPO activity more effectively as compared to PFWS where bacteria were removed. Moreover, PFW suppresses the secretion of inflammatory mediators (TNF-α, IL-4, CRP) and enhanced the anti-inflammatory (TGF-β) cytokine release. Likewise, PFW enhances the IgA level which reinforces the epithelial junction, through upregulating the expression of key junctional genes (occludin, claudin-1, ZO-1). Additionally, the transcriptional modulation of pattern recognition receptors (*TLR-2/TLR-4*) and *β-defensin-2* associated with barrier function strengthens the fact that probiotic fermented whey prevents the DSS induced infection and augments intestinal health. Furthermore, tight junction strands can become more intact as the result of a claudin-1, occludin, ZO-1 and cytoskeletal actin enhanced protein expression in PFW fed animals. Thus, this makes the probiotic fermented whey as a prophylactic functional food that could be used in the future to live a healthier life by avoiding IBD related disorders. However, human trials are of utmost necessity at this stage to validate present findings.

**Declarations**

**Declaration of Competing Interest**

The authors declare that there is of conflict of interest.

**Acknowledgements**

The authors acknowledge ICAR-National Dairy Research Institute (NDRI) Karnal, for providing the laboratory facilities for the execution of this work. We are also thankful to Indian Council of Medical Research (ICMR), New Delhi for providing required funds.

**Funding**

This work was supported by Indian Council of Medical Research (ICMR)-New Delhi (*Grant No.* 3/1/2/118/2019-(Nut) dated June 21, 2019).

**Data availability**
All data generated or analysed during this study are included in this published article.

**References**


Tables

Table 1. The criteria for disease activity index (DAI) scoring.

<table>
<thead>
<tr>
<th>Weight Loss</th>
<th>Stool Consistency</th>
<th>Bloody stool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score</td>
<td>Criteria</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>1-5%</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5-10%</td>
<td>2</td>
<td>Loose stool</td>
</tr>
<tr>
<td>11-15%</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>&gt;15%</td>
<td>4</td>
<td>Watery diarrhea</td>
</tr>
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</table>
Table 2. Oligonucleotide sequences along with their amplicon length used as primer for real time-PCR quantification

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence 5’-3’</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>F 5’GCCTCGTCCCCGATAGACAAAA3’ R 5’CAATCTCCACTTTGCCACTGC3’</td>
<td>140</td>
</tr>
<tr>
<td><strong>Claudin-1</strong></td>
<td>F 5’CCCTTCAGCAGAGCAAGGTT3’ R 5’TAGGGCAACCAAGTGCCTTT3’</td>
<td>123</td>
</tr>
<tr>
<td><strong>Occludin</strong></td>
<td>F 5’GACTCGGCTCTCACGGAAAC3’ R 5’TAGCTCGGGCATTTCTGGTG3’</td>
<td>124</td>
</tr>
<tr>
<td><strong>ZO-1</strong></td>
<td>F 5’CCTAAGACCTGTAACCATCT 3’ R 5’ CTGATAGATATCTGGCTCTC 3’</td>
<td>82</td>
</tr>
<tr>
<td><strong>hBD-2</strong></td>
<td>F 5’GCCCTTTCTACCAGCCATGAG3’ R 5’GACACAGTACCCCTACCATTGGT3’</td>
<td>151</td>
</tr>
<tr>
<td><strong>TLR-2</strong></td>
<td>F 5’AAGAGGAAGCCCAAGAAAGC3’ R 5’CGATGGAATCGATGATGTTG3’</td>
<td>199</td>
</tr>
<tr>
<td><strong>TLR-4</strong></td>
<td>F 5’ CTGCCAGAGACATTCGAGGA3’ R 5’CTGCCAGAGACATTCGAGGA3’</td>
<td>201</td>
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</table>

Table 3. Effect of feeding probiotic fermented whey on hematological parameters of mice in colitis induced animals
<table>
<thead>
<tr>
<th>Hematological parameter</th>
<th>Control</th>
<th>DSS</th>
<th>W+DSS</th>
<th>PFW+DSS</th>
<th>PFWS+DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>13.3±0.8a</td>
<td>7.82±1.6b</td>
<td>11.05±1.9ab</td>
<td>11.53±1.82ab</td>
<td>13.13±2.44a</td>
</tr>
<tr>
<td>WBC (10^3/mm³)</td>
<td>6.81±2.9a</td>
<td>12.16±4.7a</td>
<td>11.43±4.4a</td>
<td>9.45±1.87a</td>
<td>10.85±4.65a</td>
</tr>
<tr>
<td>RBC (10^6/mm³)</td>
<td>8.68±0.5a</td>
<td>4.51±0.9b</td>
<td>7.71±1.8a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte (10^3/μL)</td>
<td>2.5±0.6a</td>
<td>6.7±1.4a</td>
<td>6.36±1.2a</td>
<td>6.36±2.36a</td>
<td>6.63±4.48a</td>
</tr>
<tr>
<td>MCV (FL)</td>
<td>56.1±1.2a</td>
<td>54.95±2.5a</td>
<td>56.52±1.3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.65±1.7a</td>
<td>26.75±1.1b</td>
<td>28.83±0.4bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil (10^3/μL)</td>
<td>6.7±2.1a</td>
<td>2.9±1.4b</td>
<td>3.09±1.89b</td>
<td>2.60±1.78b</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD of five animals. Values with different alphabet are significantly different at p<0.05

**Figures**
Figure 1

Experimental design for prophylactic evaluation of probiotic *L. fermentum* fermented whey during colitis induction with dextran sulfate sodium (DSS) in mice. (A) Schematic representation of pre-treatment of mice with *L. fermentum* fermented whey with post-induction of colitis using 5% DSS for 6 days. (B) Grouping of animals. Control: healthy control mice fed with a basal diet; DSS: mice fed with a basal diet and treated with DSS; W + DSS: mice fed with a basal diet + whey and treated with DSS; PFW + DSS: mice fed with a basal diet + probiotic fermented whey and treated with DSS; PFWS + DSS: mice fed with a basal diet + probiotic fermented whey supernatant and treated with DSS.
Figure 2

Effect of probiotic fermented whey against severity of infection in DSS induced colitis model. (A) Reduction in body weight (B) Disease activity index (C) Spleen index (D) Kidney index (E) Liver index (F) Number of payer patches. Statistical analysis was done by one way ANOVA, post-hoc Tukey's test. Values are mean ± SEM (n=6) represented by vertical bars. Superscripts with unlike letters were significantly different at p<0.05.
Figure 3

**Effect of probiotic fermented whey on intestinal health in DSS induced mice.** (A) Colon weight (B) Colon length (C) Histopathological score (D) MPO activity (E) Histology of intestine Statistical analysis was done by one way ANOVA, post-hoc Tukey’s test. Data presented as mean ± SEM by vertical bars (n=5). Superscript letters indicate significant differences at p < 0.05.
Figure 4

Effect of probiotic fermented whey on secretion of cytokines and IgA in colonic tissue of DSS-induced mice. (A) TNF-α (B) IL-4 (C) CRP (D) TGF-β (E) IgA Statistical analysis was done by one way ANOVA, post-hoc Tukey's test. Values are expressed as means ± SEM represented by vertical bars (n=5). Superscript letters indicate significant differences at p < 0.05.
Figure 5

Effect of probiotic fermented whey on gut-immune functions in colonic tissue of DSS-induced mice. Transcriptional expression of (A) TLR-4 (B) TLR-2 (C) Occludin-1 (D) Claudin-1 (E) ZO-1 (F) Human beta defensin-2 (hBD-2) (G) FITC-Dextran level in serum. Values are mean ± SEM represented by vertical bars and statistical analysis was done by one way ANOVA, post-hoc Tukey's test (n=5). Superscript letters indicate significant differences at p < 0.05.
Figure 6

Representative images for immunohistochemical staining with fluorescein isothiocyanate (FITC) labelled actin filaments (phalloidin) and tight junctional proteins (occludin, claudin-1, ZO-1) in the colonic tissue of mice (Original magnification; 200X)
Figure 7

Effect of probiotic fermented whey during colitis induction on fecal bacterial count. (A) Lactobacilli (B) *E. coli* by plate count method. Values are mean ± SEM represented by vertical bars and statistical analysis was done by one way ANOVA, post-hoc Tukey's test (n=5). Superscript letters indicate significant differences at p < 0.05.