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Research Article

In vitro and *in vivo* exploration for the comparative analysis between unstimulated and inulin stimulated combinatorial probiotics

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Abstract

As per the sustainable development goals and one health programme, exploration of clinical translational outcomes of our novel probiotic combination (ABT) from ethno-medicine and conservation perspectives are important. Hence, investigation of clinical benefits of probiotics is of great value to food security and health sciences. Oxidative stress occurs due to excessive reactive oxygen species generated from superoxides and nitric oxides in our body which leads to inflammatory cascades leading to tissue damage. The immune system typically fights against infection through the generation of T- and B-lymphocytes, which lead to an adaptive immune response. In this study, we have compared the therapeutic effect of a novel combinatorial probiotic formulation "ABT" in presence of a stimulated and unstimulated state. The stimulation was added by prebiotic agent, inulin. This may stimulate the activity of "ABT" (iABT). 10^7 CFU/kg of "ABT" and "iABT" respectively that are present in a proprietary ratio and were administered orally in mice. Inulin is the prominent prebiotic that enhances the growth of the probiotics. Through *in vitro* assays, iABT has better superoxide scavenging activities, DPPH radical scavenging activities and anti-diabetic assays as compared to ABT. Further, it was validated in an *in vivo* DSS induced mice model. 3% DSS (Dextran Sodium Sulphate) was administered orally to the male BALB/c mice to mimic colitis. Weight loss is one of the important clinical symptoms in the development of colitis. Thus, both "ABT" and "iABT" both inhibits the Th17 and Th1 mediated pathway therapeutically by down-regulating different sets of immune cells.

Keywords: Dextran sodium sulphate, Inflammatory bowel disease, Inulin stimulated ABT, Oxidative stress, Unstimulated ABT

Highlights

- Administration of Dextran sodium sulphate (DSS) has aggravated the breaching of the colonic and intestinal epithelium.
- Administration of ABT has repaired the breached tissue by down-regulating the Th17 pathway.
- Administration of iABT has alleviated the tissue breaching by down-regulating the Th1 pathway.

INTRODUCTION

Lactobacillus, *Streptococcus* and *Bifidobacterium* play an important role in regulating gut health. The term reactive oxygen species (ROS) refers mainly to free radicals derived from molecular oxygen, and a few other chemically reactive molecules, which originate during the gradual reduction of molecular oxygen (Das *et al.*, 2017). Probiotic strains have been reported to scavenge hydroxyl radicals and superoxide anions and convert them into a stable compound. Probiotics can also inhibit intestinal pathogens and reduce unstable lipids, which are involved in oxidative damage. Many studies have investigated probiotic antioxidant activities, but the mechanism of action still remains unclear. Various authors have noted that exopolysaccharides, which are released by probiotic bacteria, potentially play a role in the reduction of oxidative stress. Inulin is a linear polymer polysaccharide in which D-fructose is joined by the linkage of a -(2→1) glycosidic bond in the molecular structure, and the typical terminal is a D-glucose molecule (Apolinário *et al.*, 2014; Petrovsky *et al.*, 2015). Because of the glycosidic bond types, inulin cannot be digested by digestive enzymes of monogastric animals. However, inulin can be utilized by the beneficial bacteria in the large intestine (Mudannayake *et al.*, 2015). Currently, inulin and its derivatives have been widely used in food and pharmaceutical industries (Liu *et al.*, 2015; Vassilev *et al.*, 2016; Fijan *et al.*, 2023). Various studies have been performed to investigate the antioxidant activity of inulin. In this study, titration of different inulin concentrations on the activity of our novel combinatorial probiotic ("ABT") was performed through various *in vitro* assays. Further, the activities of our novel probiotic

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combination in presence of a particular inulin concentration were validated in an *in vivo* DSS induced mice model.

Pathogenic bacteria play an important role in the entry through the leaky gut epithelial layer. The most commonly used pathogens to assess the antimicrobial activity in the publication of this issue were from the following genera or species: *Pseudomonas* spp., *Klebsiella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* spp., *Bacillus* spp. and *Salmonella* spp.

As a therapeutic strategy, antibiotics play a crucial role in the treatment of various diseases; there are adverse side effects associated with it, such as allergic reactions and gastrointestinal effects (e.g., nausea, loss of appetite, bloating, vomiting, abdominal pain and diarrhoea). The extended use of antibiotics may instigate greater antibiotic resistance not only at the individual level but also at the community, country, and regional levels.

To overcome the loopholes of antibiotics, the efficacy of ABT and iABT was validated in preclinical model through various experimental designs.

MATERIALS AND METHODS

Preparation of unstimulated and stimulated whey water

Powdered culture of "A", "B" and "T" together was added to pasteurized double toned milk, which was boiled and cooled to room temperature, followed by overnight incubation at 42°C as mentioned in (Mitra *et al.*, 2022). The collected whey water that contains only the bacterial formulation "ABT" remains in an unstimulated state. Similarly, for the stimulated state, both ABT and inulin were added and incubated overnight at 42°C. Titration of different concentrations of inulin (HIMEDIA, GRM103-25G) (0.5%, 1% and 2%) was considered to study its antimicrobial and anti-oxidative activity. Different concentrations of inulin were added to the milk along with probiotic powder ("ABT"). Further, both ABT and iABT formed above the fermented milk (curd), were strained through nylon mesh and collected in tubes for performing assays.

Sources of pathogenic bacteria

The most common food-borne pathogens (*Staphylococcus aureus* was collected from the Microbiology Research Lab, Department of Microbiology, University of Calcutta), *Vibrio cholerae* and *Bacillus cereus* strains were collected from the Virology division of ICMR - National Institute for Research in Bacterial Infections. The bacterial stab was brought to the research laboratory (Immunology and Regenerative Research Unit, Department of Immunology) in a sealed-packed box to prevent contamination.

Determination of antibacterial activity of the probiotics

To examine the efficacy of ABT and iABT against different tested bacteria, the agar well diffusion method on Luria Agar (HIMEDIA-M1151F 500G) plate was performed. According to this method, a loop full culture of the tested bacteria (*Staphylococcus aureus*, *Vibrio cholerae* and *Bacillus* cereus) was inoculated into the appropriately labelled sterile test tubes containing Luria broth (LB) (HIMEDIA- M1245-500G) to culture the bacterial suspension and the bacterial lawn was prepared onto the surface of the LA media using 100 µL of pathogenic bacteria. Then, wells were made on the Luria agar media, and 20 µL of sample (ABT and iABT) was poured into each well. After incubating overnight at 37°C, the presence of a clear zone around the sample solution (if any) was analyzed by measuring the clearance zone with the ruler.

Anti-oxidant activities

DPPH free-radical scavenging assay: DPPH radical scavenging ability was measured with some modification (González-Palma *et al.*, 2016). The 750 µL of sample (unstimulated and stimulated ww) was mixed with 750 µL of 0.3 mM DPPH (2,2-Diphenyl-1-Picrylhydrazyl extrapure) solution and incubated for 30 min at 37°C in the dark to determine the absorbance value at 517 nm using spectrophotometer (Varioskan LUX). Blank reaction was also performed using methanol as blank (SRL-132977) and 0.3 mM DPPH. A stock solution of 3 mM DPPH was prepared in methanol. The DPPH-free radical scavenging activity was counted as follows:

DPPH radical scavenging activity % = (Blank-OD_S)/ Blank × 100, where blank represents the Abs of the control group replaced the sample solution with methanol; OD_S is the Abs of the test sample. Ascorbic acid was used as positive control.

Superoxide radical scavenging assay: 250 mL of 50 mM sodium carbonate (Merck 17844), 50 mL of 0.1 mM EDTA (Promega V4321) and 0.1 mM HaH (Hydroxylamine hydrochloride SRL 084784) was vortexed and incubated for 10 mins. 125 mL of sample (unstimulated and inulin stimulated cell free supernatant) was added. Finally, 100 mL of NBT (Nitroblue Tetrazolium SRL 48898) was added to the solution and incubated for 5 mins at room temperature. OD was measured at 560 nm using a spectrophotometer (Varioskan LUX).

Superoxide scavenging activity % = (Blank-OD_S)/ Blank × 100, where blank represents the Abs of the control group replaced the sample solution with blank, and OD_S is the Abs of the test sample. Ascorbic acid was used as positive control.

Anti-diabetic activities

Alpha-amylase inhibition assay: In this method, α–amylase enzyme converts the starch into maltose. 50 mL of samples was incubated with 50 mL of 0.5 mg/ mL of a-amylase (HIMEDIA, RM638) at 37°C, for 10 min. 100 mL of DNSA (SRL13313) was added and boiled at 100°C for 5 mins. It was further cooled down. Finally, the volume of reaction mixture was made upto 1 mL by distilled water. Absorbance was measured at 540 nm. Acarbose (Glucobay 25) (Bayer Zydus Pharma) was used as the standard chemical for this assay.

Protocol followed for *in vivo* validation

Induction of disease and treatment with probiotics: Male BALB/c mice (6 weeks old, 20-25 g) were divided into four groups (n = 4): Control, DSS, ABT and iABT. The mice of both disease groups (DSS) and therapy groups (ABT, iABT) were administered with 3% DSS (in autoclaved water) orally, on days 0, 3, 5 and 7. ABT, prepared overnight, containing approximately $1.8*10^6$ CFU of ABT, translating to a dose of 10^7 CFU/kg probiotics, and inulin stimulated whey water (iABT), containing approximately $2*10^6$ CFU of ABT, translating to a dose of 10^8 CFU/kg probiotics was administered orally to the mice groups (ABT, iABT), on days 5, 7, 9, 11 and 13.

Collection of tissues: Colon tissues were taken, washed with PBS and stored at -20°C for oxidative stress assay. For histological studies through cryosection, colon tissues were collected in 4% paraformaldehyde. The tissue was stained with haematoxylin and eosin for studying the morphology and alcian blue staining for identifying the number of goblet cells. For gene expression studies, colon tissues were stored in RNAlater solution (Ambion, Inc.) at -20°C until use.

Measurement of body weight: The body weight of the mice of all 4 groups was recorded throughout the regimen.

Visual observation of colon: Colon of mice from different groups were immediately isolated after the sacrifice. The colon was excised between the ileocaecal junction and the proximal rectum and was placed on a non-absorbent surface, and its length was measured with a ruler without stretching and damaging the colon.

Morphological observation of colon tissue: According to the protocol used by Biswas *et al*. (2024), colon tissues were processed in 15% sucrose and 30% sucrose overnight. After the sucrose was fully imbibed in the tissue, it was embedded in Tissue Freezing media (Leica) and cut into 5 µm sections in a cryocut instrument (Leica CM1860). The Maeyer's albumin fixed sections were stained with hematoxylin (Merck- DB1DF71034) and Eosin (Nice- E30971) to assess overall morphological architecture and alcian blue staining (HIMEDIA-RM471) to identify mucus containing goblet cells of the colon. The stained sections were observed under a light microscope at 4X and 10X (Olympus BX41) magnification (Mitra *et al*., 2022).

Determination of oxidative stress markers (NO and MDA) and anti-oxidative markers (SOD and Catalase): As mentioned in Biswas *et al*. (2024), nitric oxide content (NO) in colon and intestine was determined by using Griess reagent, which comprises of (1% sulphanilamide (SRL: 1949107), 0.1% NED (HIMEDIA-RM1073), 5% ortho-phosphoric acid (MERCK-AG8A580394) was added with sample (supernatant of colon and intestine), incubated for 10 mins and Optical Density (OD) was measured at 540 nm. A standard curve was prepared using 0.1M $NaNO_2$ (MERCK-17543).

Likewise, Superoxide dismutase (SOD) activity of both these tissues was determined by using 50 mM Na_2CO_3 (MERCK-17844) 0.1 mM EDTA (Ethylene diamine tetra acetic acid) (Promega-000014215), 5 mM NBT (Nitroblue Tetrazolium (SRL-48898)) and 1mM Hydroxylamine hydrochloride (HAH) (SRL-84784) along with the sample, incubated for 5 min and OD was measured at 560 nm (Aminabee *et al*., 2020; Biswas *et al*., 2024). A standard curve was prepared using similar concentration of Superoxide dismutase (SOD) tablet (NUTRACEUTICAL: HGS AESTHETICS: ALC-023) as mentioned by Biswas *et al*. (2024).

Catalase activity was measured by using a 10 mM H_2O_2 reagent (MERCK-CJ9C90644) dissolved in 50 mM phosphate buffer along with the sample, and OD was measured at 240 nm at an interval of 10 mins. The optical density (OD) of all the above-mentioned assays was measured in a spectrophotometer (Varioskan LUX) (Nagababu *et al*., 2010; Biswas *et al*., 2024).

Gene expression by reverse transcriptase PCR (RT-PCR): The protocol for RNA extraction from colon was followed from Biswas *et al*., 2024. Concentration of RNA was measured by re-suspending the pellet in nuclease free water in Nanodrop (Jenway, UK). RT-PCR was performed from the extracted RNA to produce cDNA

using Verso cDNA synthesis kit, Life Technologies, USA. cDNA was prepared by following the below mentioned cycle using 2 mg RNA (Table 1) (Mitra and Banerjee, 2022; Mitra *et al*., 2022).

Table 1. Protocol for PCR amplification

Temperature	Time	No. of cycle
95ºC	5 min	1 cycle
95ºC	45 sec	30 cycles
TºC	30 sec	
72ºC	45 sec	
72ºC	10 min	1 cycle
4ºC	Infinite	

Since the DSS-induced IBD model follows mainly Th1 and Th17 mediated immune responses, it involves the role of TNFα (Tumor Necrosis Factor), IFNλ (interferon gamma), IL17 (Interleukin 17) Claudin1 and ZO1 (Zonula Occludens) in degeneration of intestinal epithelium by disruption of barrier function integrity. Thus, the expression of these genes was assessed by PCR (Table 1) using specific primers (Table 2), with GAPDH as the housekeeping gene and a 100 bp marker (100-2000 bp; Proxy B, SRL 84628). These PCR products were run in 1.2% agarose gel (G-Biosciences, USA). Bands were observed under UV light in a gel-doc system (Biorad Chemidoc Imaging System), and the band intensities were determined using Image J Software.

Statistical analysis

All data (represented as Mean ± SD) were analyzed, and statistical significance ($p<0.05$) was calculated by student's t-test in excel. Comparison of multiple experimental groups was performed by 2-way ANOVA. For the *in vitro* cell-free studies, * has been used to denote significance with respect to unstimulated ABT. In the following *in vivo* assays, *has been used to denote significance with respect to control groups, and # has been used to denote significance in comparison to DSS-treated mice groups.

RESULTS

***In-vitro* studies**

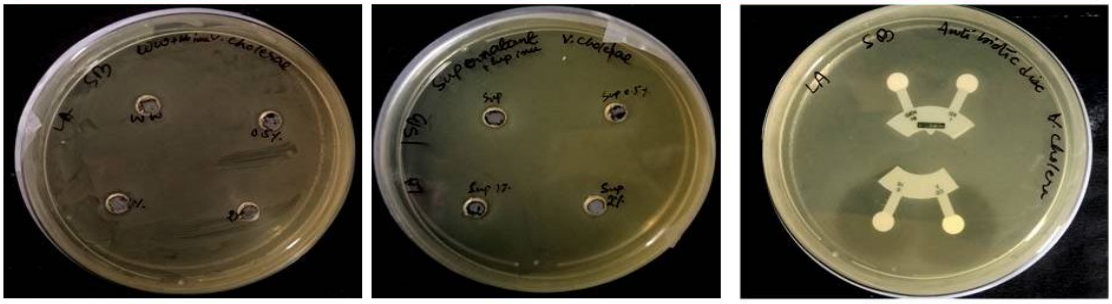
Determination of antimicrobial activity: It may be inferred that both ABT and iABT have significant antibacterial effect against several pathogenic strains of bacteria, including *Vibrio cholerae*, *Staphylococcus aureus* and *Bacillus cereus*. All the strains, *S. aureus*, *V. cholerae* and *B. cereus* can alter the overall microbial metabolic profiles by damaging the intestinal epithelium. Both ABT and iABT have inhibited the growth of intestinal breach inducing pathogens, *V. cholerae* and *S aureus*. Not only the cellular component of probiotic, but also the secretome obtained from the probiotic combination have an important role in regulating the growth of all the three pathogens (Fig. 1).

Anti-oxidant activities

DPPH free-radical scavenging assay: DPPH is a free radical that shows a purple colour in methanol solution. Antioxidants scavenge this radical, which upon reduction causes a decolorization to yellow. Likewise, different concentrations of inulin-stimulated whey water possess DPPH scavenging activity. Unstimulated whey water (ABT) shows higher DPPH scavenging activity (58.96%), 0.5% inulin stimulated ABT (iABT) shows (50.35%), ww-1% inulin (58.36%) and ww-2% inulin (44.34%) but not as high as unstimulated whey water. Out of different inulin-based whey water, 1%

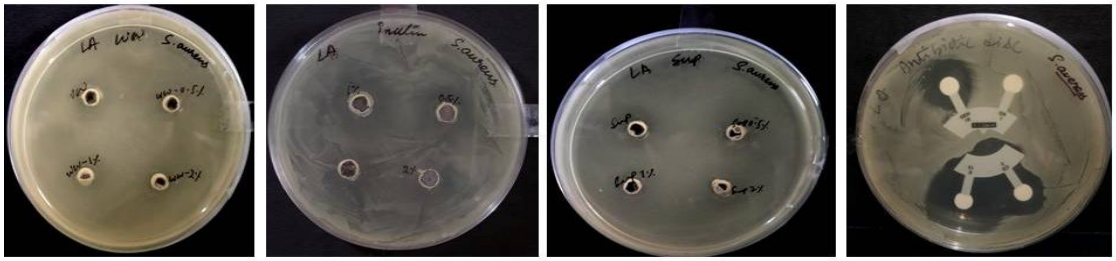
Table 2. Primers of specific genes

Gene	Primer sequence	Tm (°C)	Product size
GAPDH	F 5′ - GAGGGGCCATCCACAGTCTTC 3′ R 5′ - CATCACCATCTTCCAGGAGCG 3′	62.75	357 bp
Claudin 1	F 5′ AGGTCTGGCGACATTAGTGG 3′ R 5′ CGTGGTGTTGGGTAAGAGGT 3′	59.35	204 bp
ZO-1	F 5′ ACTCCCACTTCCCCAAAAAC 3′ R 5′ CCACAGCTGAAGGACTCACA 3′	58.32	166 bp
IFNγ	F 5′ AGCGGCTGAACTCAGATTGTAG 3′ R 5′ GTCACGTTTTCAGCTGTATAGGG 3′	62.9	247 bp
TNFα	F 5′ GGCAGGTCTACTTTGGAGTCTTGC 3′ R 5′ ACATTCGAGGCTCCAGTGAATTCGG 3′	64.62	300 bp
NFkB	F 5′ AGAGGATTTCGATTCCGCTA 3′ R 5′ CGTGAAGTATTCCCAGGTTG 3′	57.06	274 bp
IL17	F 5′ CAGCAGCGATCATCCCTCAAAG 3′ R 5′ CAGGACCAGGATCTCTTGCTG 3′	60.88	302 bp



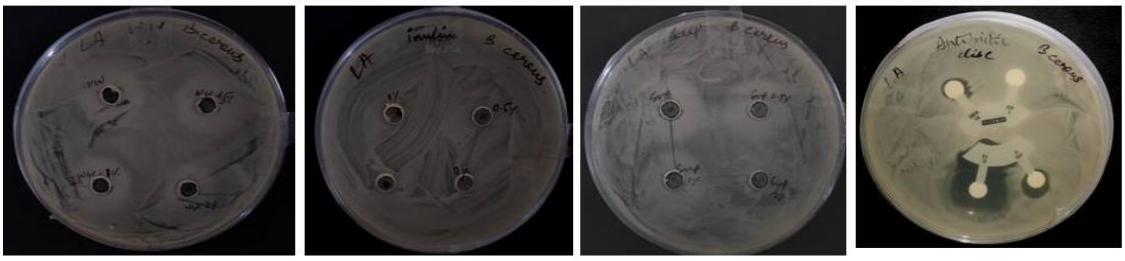
Pathogen: *Vibrio cholerae*					
Sample	Zone of inhibition (cm)	Sample	Zone of Inhibition (cm)	Sample	Zone of Inhibition (cm)
ww	0.2±0.00	0.5% inulin	No inhibition	Sup	0.13±0.057
WW-0.5% inulin	0.23±0.05	1% inulin	No inhibition	SUP-0.5%	No inhibition
WW-1% inulin	0.37±0.05	2% inulin	No inhibition	SUP-1%	0.3±0.00
WW-2% inulin	No inhibition			SUP-2%	No inhibition

Pathogen: *Vibrio cholerae*	
Antibiotic disc	Zone of inhibition (cm)
Gen	No inhibition
Oxa	No inhibition
CD	2.37±0.17
E	2.03±0.05



Pathogen: *S aureus*					
Sample	Zone of inhibition (cm)	Sample	Zone of inhibition (cm)	Sample	Zone of inhibition (cm)
ww	0.33±0.057	1% inulin	1.50±0.00	Sup	0.57±0.115
WW-0.5% inulin	No inhibition	0.5% inulin	1.67±0.28	SUP-0.5% inulin	0.40±0.1
WW-1% inulin	No inhibition	2% inulin	No inhibition	SUP-1% inulin	No inhibition
WW-2% inulin	No inhibition			SUP-2% inulin	No inhibition

Pathogen: *S aureus*	
Antibiotic disc	Zone of inhibition (cm)
GEN	0.5±0.00
Oxa	No inhibition
CD	0.57±0.15
E	2.2±0.52



Pathogen: *Bacillus cereus*					
Sample	Zone of inhibition (cm)	Sample	Zone of inhibition (cm)	Sample	Zone of inhibition (cm)
ww	No inhibition	1% inulin	No inhibition	Sup	0.27±0.0577
WW-0.5% inulin	No inhibition	0.5% inulin	No inhibition	SUP-0.5% inulin	No inhibition
WW-1% inulin	No inhibition	2% inulin	No inhibition	SUP-1% inulin	0.23±0.057
WW-2% inulin	No inhibition			SUP-2% inulin	No inhibition

Pathogen: *Bacillus cereus*	
Antibiotic disc	Zone of inhibition (cm)
GEN	1.33±0.15
Oxa	No inhibition
CD	2.17±0.65
E	2.43±1.28

Fig. 1. Growth of bacterial strains, *Vibrio cholerae*, *Staphylococcus aureus*, and *Bacillus cereus* was inhibited by ABT and iABT along with their secretome. Inhibition was prominent against *V. cholerae* by unstimulated ABT, 0.5% iABT and 1% iABT and *S. aureus* only by unstimulated ABT. Bacterial secretions (secretome) also have the antimicrobial property. Inhibition was observed against *V. cholerae* and *B. cereus* by unstimulated and 1% inulin stimulated secretome (Sup and Sup-1%inu). Inhibition was observed against *S. aureus* by unstimulated and 0.5% inulin stimulated supernatant (secretions) (Sup and Sup-0.5%inu).

inulin-stimulated whey water (iABT) has reduced DPPH scavenging activity as compared to unstimulated whey water (ABT) by 1 fold. Whereas, 0.5% iABT and 2% iABT showed 1.387 and 1.7 folds lesser DPPH scavenging activity than ABT. Thus, out of different stimulated whey water, 0.5% iABT shows a significantly similar DPPH scavenging activity (with a p-value of 0.04), 1% iABT shows significance with a p-value of 0.012 as compared to the stimulated state (ABT). 2% iABT shows its significance with a p value of 0.025. On the basis of significance, 1% iABT has a similar significance as compared to ABT (Fig. 2).

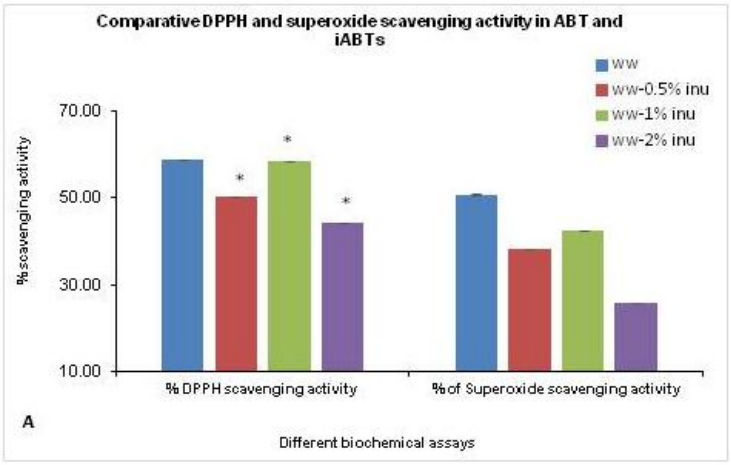


Fig. 2. Unstimulated whey water shows better anti-oxidative activities as compared to 1% inulin stimulated whey water. 1% inulin stimulated whey water shows significant anti-oxidative activity ($p<0.05$), as compared to other inulin stimulated whey water. 1% inulin stimulated whey water shows a decreased DPPH scavenging activity as compared to whey water and a decreased superoxide inhibition activity as compared to whey water.

Superoxide radical scavenging assay: Unstimulated whey water (ABT) has shown an increased superoxide inhibition (50.73%) as compared to inulin stimulated whey water. 0.5% iABT, 1% iABT and 2% iABT show a certain superoxide inhibition activity (38.09%, 42.34% and 25.7%). All the inulin-stimulated ABTs (0.5%, 1%, and 2% iABT) shows a 1.33, 1.19, 1.97 fold change with respect to ABT. All iABTs have a p-value greater than 0.05. This fold change shows that 1% iABT shows a lesser difference in the superoxide inhibition activity as compared to ABT. As per the performed two-way ANOVA test, it may be inferred that an alternative hypothesis can be considered as a p-value less than 0.05. Superoxide inhibition activity is different in different samples as it shows a p-value of 0.01. However, differences in superoxide and DPPH scavenging inhibition are not significantly evident among different samples as the F statistic value is greater than the F critic value.

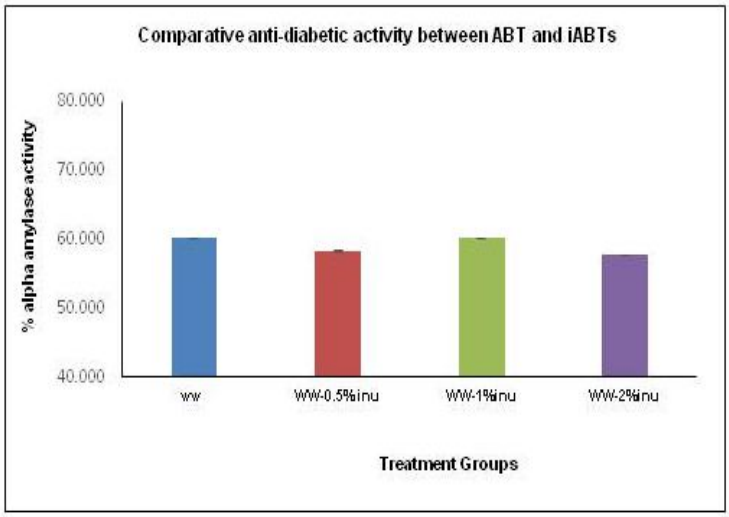


Fig. 3. 1% inulin stimulated whey water shows better anti-diabetic activity as compared to other inulin stimulated whey water.

Anti-diabetic activities

Alpha-amylase inhibition assay: The α-amylase, a key enzyme of carbohydrate metabolism, was efficiently inhibited by both unstimulated and stimulated whey water. ABT shows 60.215% of amylase inhibition activity. 0.5% iABT shows 58.35% inhibition, 1% inulin-stimulated whey water (1% iABT) shows 60.178% inhibition and 2% iABT shows 57.687% inhibition, out of which 1% iABT shows the highest inhibition among different iABTs. There is a fold change difference among different types of 0.5%, 1% and 2% iABTs, which is 1.032, 0.99, and 1.044 folds lesser inhibition activity, respectively. From the above observation, it may be concluded that 1% inulin-stimulated whey water (iABT) has better anti-diabetic activity as compared to ABT ($p<0.05$) (Fig. 3). As per the performed two-way ANOVA test, it may be inferred that a null hypothesis can be considered as p value is greater than 0.05. Alpha amylase inhibition activity is different in different samples but not significant as the p-value is 0.342, which is greater than 0.05, though the F statistic value is greater than the F critic value.

From the above performed *in vitro* cell free assays, it may be concluded that out of all titrable concentrations,

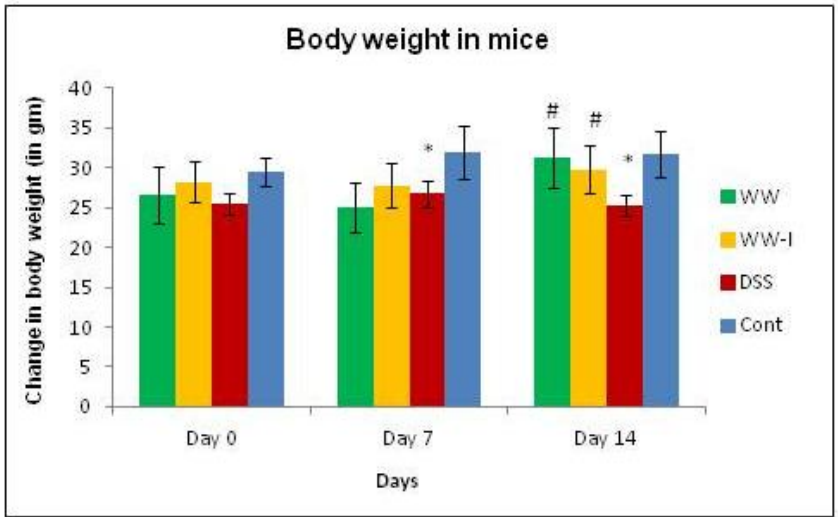


Fig. 4. Body weight in both days 7 and 14 of DSS groups has decreased with respect to control. On days 7 and 14, WW has increased the body weight with respect to DSS. Inulin-stimulated ww has increased the body weight with respect to DSS.

1% inulin based whey water has shown a significant anti-diabetic and DPPH scavenging activites. Further, the regulatory pathway of unstimulated whey water WW (ABT) and 1% WW-I (iABT) was validated in an *in vivo* condition through pre-clinical mouse model.

Results from in vivo experiment

Therapeutic administration of ABT and iABT have alleviated the symptoms of colitis in mice: Weight loss is one of the most prominent clinical symptoms in IBD. Administration of DSS has significantly reduced the body weight by 1.19 and 1.25 folds, respectively, as compared to control on day 7 (with a p-value of 0.03) and 14 (with a p-value of 0.012). Therapeutic administration of unstimulated and 1% inulin stimulated whey water (ABT, iABT) has restored the body weight as compared to disease groups throughout the disease model. ABT has restored the body weight significantly by 1.02 and 1.23 folds ($p<0.05$) compared to the DSS group on day 7 and 14, whereas, in 1% iABT groups, the body weight was restored significantly with respect to DSS by 1.03 and 1.17 folds on day 7 and 14 (p: 0.04), respectively. Thus, it may be inferred that mice groups, where both ABT and 1% iABT show a significant improvement in body weight as compared to DSS-treated groups (Fig. 4).

Therapeutic administration of ABT and iABT have healed the colon length of mice: The length of the colon was recovered in therapeutic groups (ABT, iABT) as compared to the disease group (DSS). DSS induced colitis mice shows a reduction in colon length by 1.12 fold with respect to control, whereas, the therapy groups (ABT) have increased their colon length by 1.078 fold with respect to diseased groups and inulin stimulated whey water (iABT) groups have also shown a restoration in colon length by 1.11 fold with respect to DSS groups (Fig. 5). As per the above performed two-way ANOVA

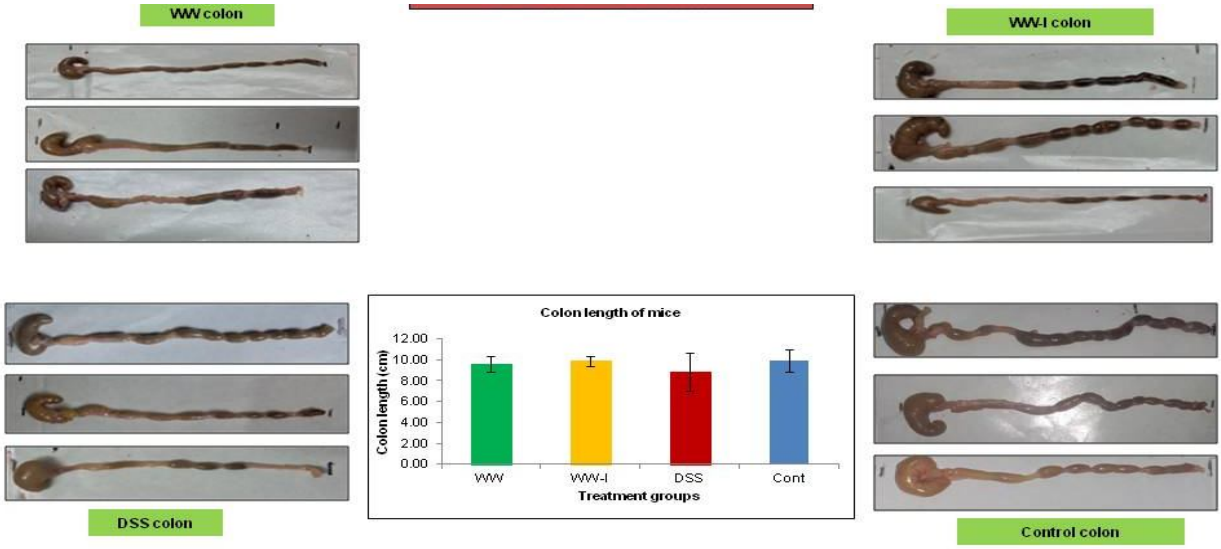


Fig. 5. Colon length of DSS groups was decreased with respect to control, whereas, therapeutic groups (ABT) and the inulin stimulated whey water (iABT) have increased the colon length with respect to DSS.

test, it may be inferred that an alternative hypothesis can be accepted as p-value is less than 0.05 (0.02) and F statistic value is greater than F critic value. This shows that there is a significant comparative difference in the colon length of mice among different groups.

Therapeutic administration of ABT and iABT have restored morphological structure of colon: The epithelial architecture of colon was damaged in DSS groups, which was observed by hematoxylin and eosin staining, whereas, the therapeutic groups (ABT and iABT) have restored the architectural damage caused by DSS (Fig. 6).

The mucus layer along the GI tract, formed by mucin secretion from goblet cells, is most important for mucosal defense. In IBD, the degeneration of colonic mucosa has resulted in the depletion of the mucus layer and a reduction in the number of goblet cells. Goblet cells were depicted by alcian blue staining. The depleted amount of goblet cells was successfully restored by the administration of therapies ((ABT and iABT) to disease-induced mice (Fig. 7).

Therapeutic administration of ABT and iABT have healed the excessive oxidative damage in DSS induced colon: In IBD, oxidative stress is one of the important factors that lead to a degenerated colonic layer. In colon, oxidative stress results in significant increase in NO levels (p: 0.05) in colitis by 1.59 fold with respect to control, whereas, the therapeutic groups (ABT; iABT) have exhibited a significant reduction in NO level by 2.65 and 2.92 fold with respect to DSS. Therapy groups (iABT) have shown a significant inhibition of generation of nitric oxide ($p<0.05$). This shows that (iABT) works by primary inhibition of oxidative stress (Fig. 8B). As per the two way ANOVA test, it may be inferred that a null hypothesis can be accepted as p-value is greater than 0.05 (0.965) and F statistic value is lesser than F critic value. This shows that there is no significant comparative difference in nitric oxide (NO) content among different groups of mice.

Anti-oxidative enzymes (SOD and catalase) work in a simultaneous fashion (Fig. 8A, Fig. 8C). SOD plays an important role in scavenging superoxides to produce hydrogen peroxides, and in turn, catalase acts on

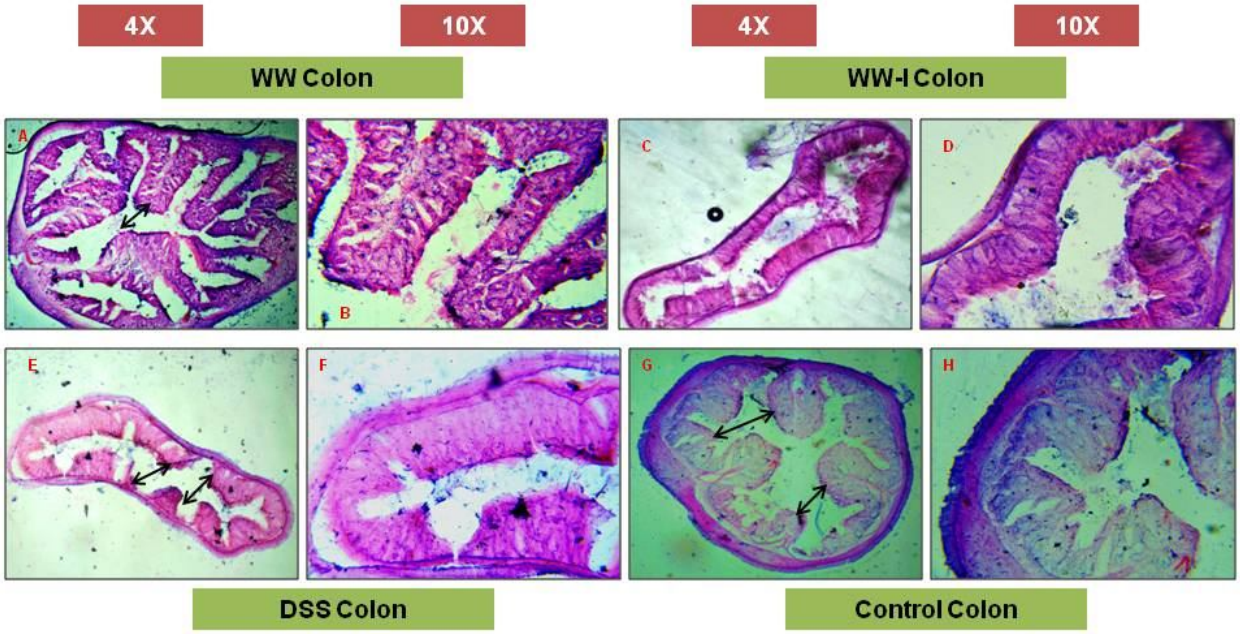


Fig. 6. A and B: (4X and 10X): Both the magnification shows that ww colon shows an almost recovered invaginations as compared to DSS induced colon and the gut lumen is smaller than diseased groups as depicted through H&E staining. C and D: (4X and 10X): Both magnification shows that inulin-stimulated whey water also has the capacity to recover the invaginations of the colonic epithelium. Compared to DSS, these pictures are captured using a light microscope. The black colour shows the length of the gut lumen. E and F (4X and 10X): The DSS induced acute colitis has shown a degenerated colonic epithelium and reduced gut lumen as compared to DSS. G and H (4X and 10X): Control colon shows a larger invagination and the gut lumen is smaller than diseased groups as depicted through H&E staining. These pictures are captured in the light microscope.

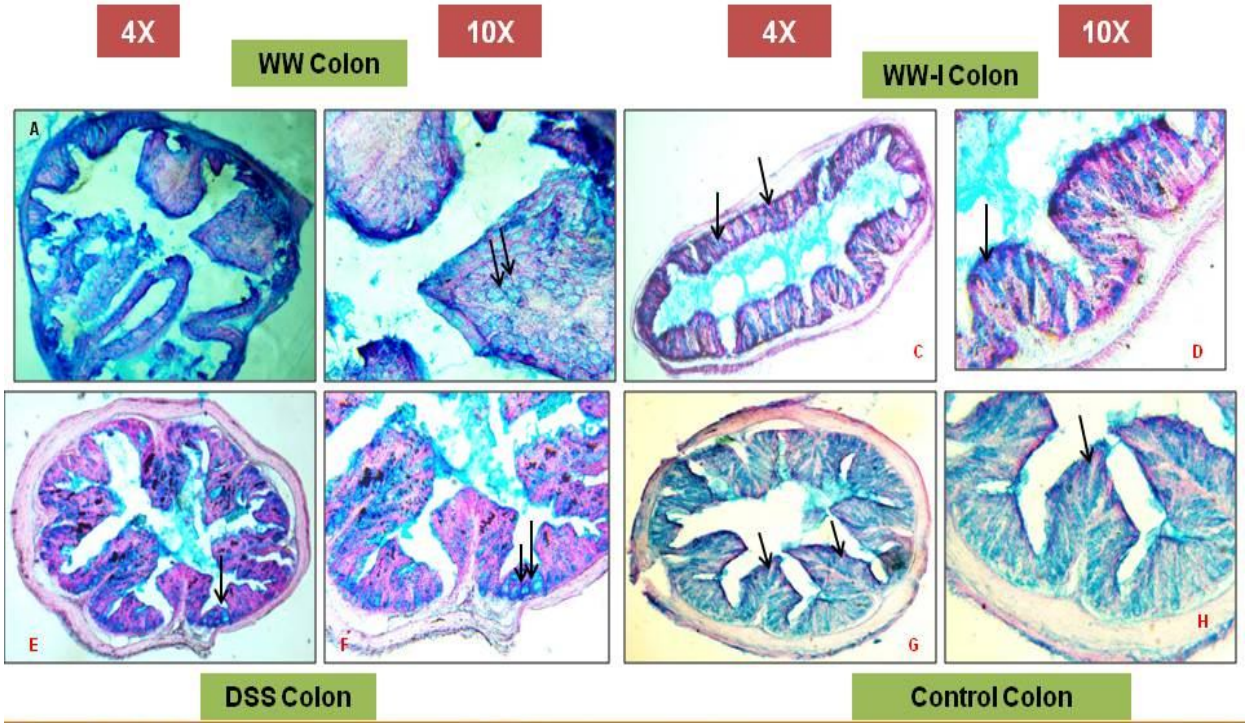


Fig. 7. A and B: (4X and 10X): Both the magnification show that the alcian blue stained ww colon shows a recovered number of goblet cells as compared to the induced colon and the area of the gut lumen is smaller than diseased groups, which depict a reduced degeneration of intestinal layer. C and D: (4X and 10X): Both magnification shows that inulin-stimulated whey water (WW-I) has the capacity to recover the invaginations of the colonic epithelium similar to that of whey water (WW). E and F (4X and 10X): Number of goblet cells has reduced as compared to control and therapy groups. G and H: (4X and 10X): Control colon tissues show an optimum no. of goblet cells, which, in turn, will maintain the mucus layer of the colon. These pictures are captured in the light microscope. Black arrows show the presence of goblet cells.

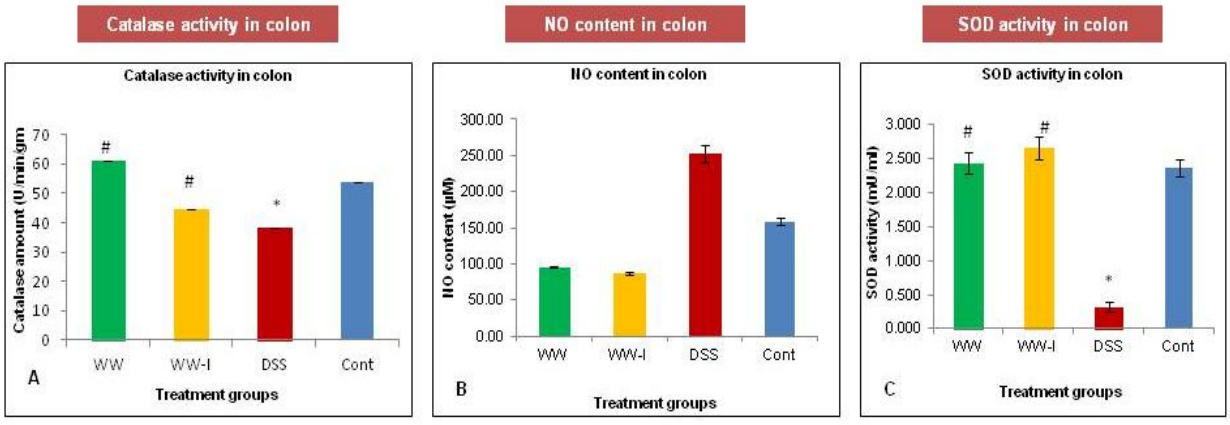


Fig. 8. A. Catalase level has significantly decreased in the colon. Therapeutic groups (WW: ABT and WW-inulin: iABT) have significantly restored the catalase levels in the colon as compared to DSS B. NO content has increased in the colon of DSS groups with respect to control, whereas ww (ABT) and ww-inulin (iABT) have decreased the NO content with respect to DSSC. DSS decreases the SOD content in the colon with respect to control, whereas ww (ABT) and ww-inulin(iABT) have increased the SOD content as compared to DSS (*: $p < 0.05$, compared to control; #: $p < 0.05$, compared to DSS).

hydrogen peroxides to form H_2O. Formation of water may help in repairement of intestinal tissue damage. DSS-induced colitis results in decreased catalase and SOD activity as compared to control by 1.415 fold (with a p-value of 0.0042 and 7.38 folds (with a p-value of 0.02), respectively. Activity of SOD in colon of therapeutic mice groups (ABT, iABT) were enhanced with respect to DSS by 7.51 (with a p-value of 0.02) and 8.31 folds (with a p-value of 0.009), respectively. Catalase activity in the colon of (ABT, iABT) was significantly increased with respect to DSS by 1.61 (with a p-value of 0.0069) and 1.17 folds (with a p-value of 0.0084), respectively. Unstimulated ABT works by upregulating the catalase activity, which enhances the formation of water that has inhibited the breaching of intestinal layer.

Therapeutic administration of ABT and iABT have repaired the imbalance of pro-inflammatory cytokines and junctional proteins in colon: Inflammation that occurs in IBD results in up-regulation of pro-inflammatory Th1 markers (IFNγ, TNFα, NFkB and IL17), results in degradation of barrier function integrity of colon by reducing the expression of Claudin 1 and ZO1. Our study has concluded that there is a significant down-regulation of junctional proteins like ZO-1 and Claudin-1 in DSS groups by 2.8 (with a p-value of 0.00014) and 1.35 folds (with a p-value of 0.007) respectively with respect to DSS (Fig. 9). On the contrary, colonic barrier disruption was healed by therapeutic groups (ABT, iABT) by up-regulating the junctional proteins like ZO-1 by 1.74 and 1.23 folds respectively (with a p-value of 0.0005) with respect to DSS and Claudin-1 by 1.32 and 1.505, respectively with respect to DSS. Moreover, we have found an upregulation of cytokine markers (IFNγ, TNFα, IL17 and NFkB), which is significantly increased in DSS induced colon by 2.62 (with a p-value of 0.00025), 1.07 (with a p-value of 0.012), 1.13 (with a p-value of 0.024) and 6.411 folds (with a p-value of 0.00005) with respect to control. On the other hand, ABT and iABT) groups have decreased the expression of pro-inflammatory cytokine IFN-γ by 1.36 (with a p-value <0.05) and 1.5 fold (with a p-value <0.05) with respect to DSS, TNFα (by 1.56 and 2.02 folds with respect to DSS, with a p-value <0.05), NFkB 4.98 and 8.42 folds with respect to DSS, (with a p-value <0.05) and IL17 (by 2.2 and 1.91 folds with respect to DSS, with a p-value of 0.05). Thus, it may be inferred that ABT and iABT) has healed DSS induced colitis by Th17 pathway and Th1 pathway, respectively (Fig. 9).

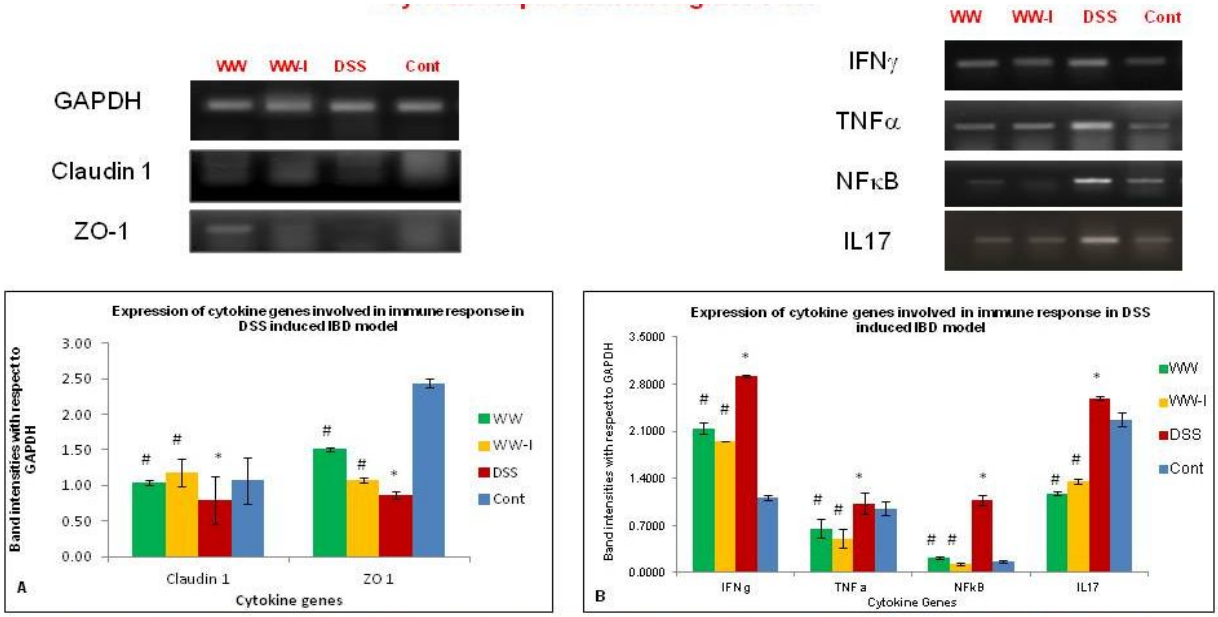


Fig. 9. A. In colon, gene expression of Claudin1 has significantly decreased in DSS as compared to control groups, whereas, both unstimulated whey water (WW) and inulin stimulated whey water (WW-I) have significantly increased the expression. In colon, gene expression of ZO1 has significantly decreased in DSS as compared to control groups whereas, both unstimulated whey water (WW) and inulin stimulated whey water (WW-I) have significantly increased the expression with respect to DSS (*: p<0.05, compared to control; #: p<0.05, compared to DSS). B. In colon, gene expression of IFNγ, TNFα, NFkB and IL17 have significantly increased in DSS as compared to control groups, whereas, both unstimulated whey water (WW) and inulin stimulated whey water (WW-I) have significantly reduced the expression with respect to DSS. (*: p<0.05, compared to control; #: p<0.05, compared to DSS). Band intensities have been calculated with respect to housekeeping gene, GAPDH.

DISCUSSION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that is characterized by an anomalous immune response in the gastrointestinal (GI) tract. Chemically induced colitis (DSS) has been extensively used to investigate the molecular mechanism of colitis that has been mimicked in male mice. Thus, a 3% DSS induced colitis has been developed, and our novel probiotic formulation (ABT and iABT) in the form of whey water, and was applied as therapy to study the therapeutic activity against DSS induced colitis. DSS induced degeneration are accompanied by histopathological modification that includes symptoms which are eventually expressed as bloody diarrhea. This considerably increases the output of all proinflammatory cytokines in the intestine that increases the degeneration of intestinal and colonic mucosa (Fig. 10) (Meers *et al.*, 2018; Fijan, 2023).

Here comes the beneficial role of unstimulated whey water (ABT) and inulin stimulated whey water (iABT). Body weight and colon length were significantly reduced in both ABT and iABT. Experimental results demonstrated that in colitis, due to rigorous diarrhoea and water loss, the body weight of these mice became the lowest, and the colon lengths were the shortest. iABT groups have better healing activity as they restore the colon length as compared to DSS. Moreover, on the basis of morphology, it may be concluded that administration of ABT (10^7 CFU/mL) and iABT (10^8 CFU/mL) had also significantly healed the degeneration of the colonic epithelium caused by DSS, which may be vividly evidenced through histological staining. Due to tissue damage, lesser mucus secretion from the goblet cells has enhanced the breaching of intestinal epithelium. Therapy groups (ABT and iABT) restored the mucous layer compared to DSS. Continuous breaching of colon tissue due to DSS had led to an increase in the level of nitric oxide (NO) that generates oxidative stress. This in turn had decreased the anti-oxidative activity. Reduction of SOD (superoxide dismutase) activity has activated the NFêB pathway in disease groups. This activates the T-cell mediated cytokine pathway. In healthy condition, superoxide dismutase (SOD) enzymes convert superoxides generated in our body, into hydrogen peroxides. In turn, catalase (CAT) scavenges this hydrogen peroxide into water molecules. This

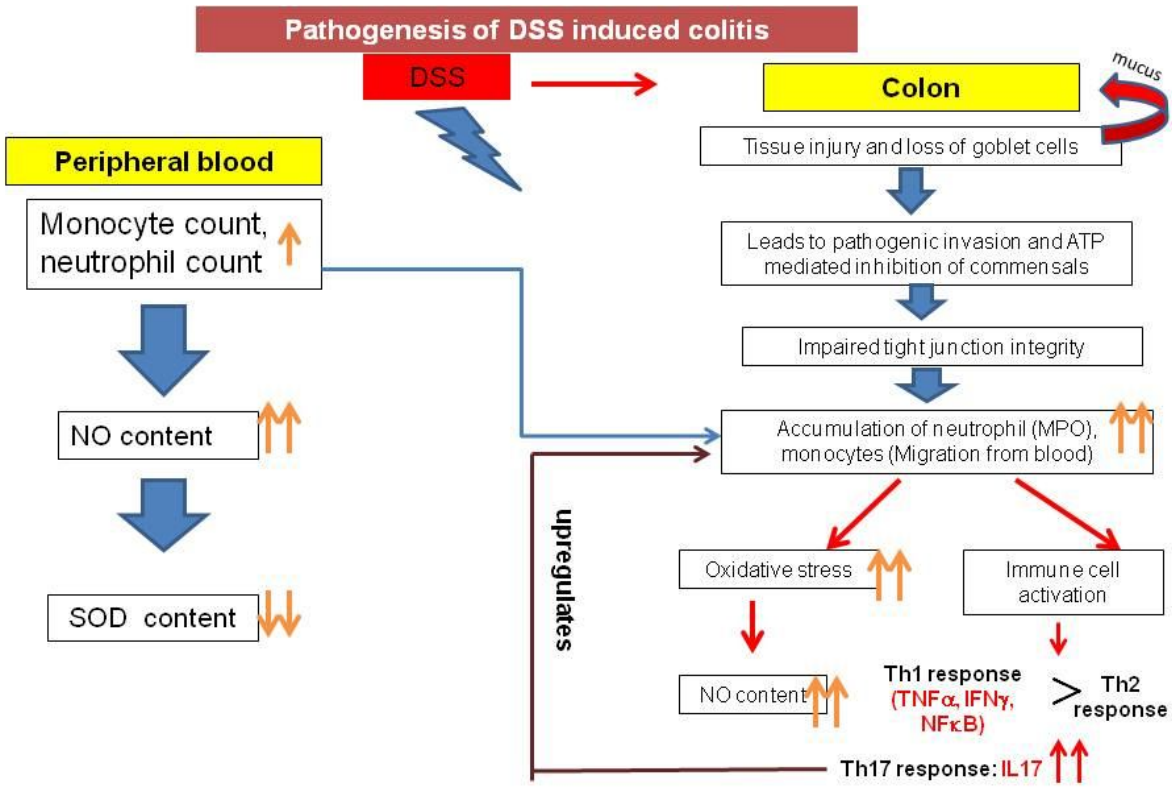


Fig. 10. Hypothetical pathway of DSS up-regulating the Th1 and Th17 immune response through CD4+ mediated and macrophage-neutrophil mediated pathway, respectively

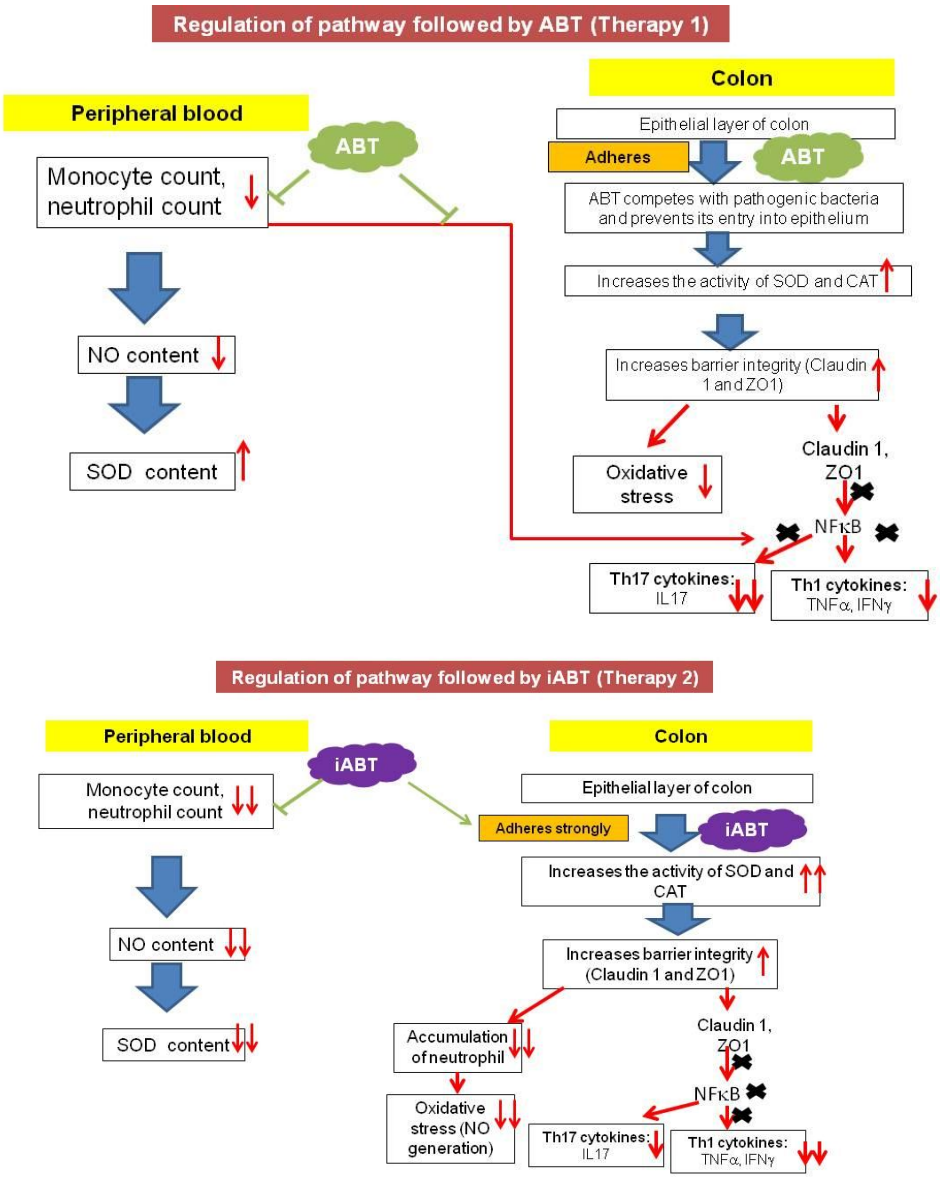


Fig. 11. Hypothetical pathway of ABT and iABT by down-regulating the Th17 and Th1 immune response, respectively.

mechanism is hindered in the presence of DSS (dextran sodium sulphate). Therapy-treated mice groups (ABT) have an important role in regulating CAT (catalase) activity. It can significantly scavenge the peroxides formed due to oxidative stress. Both ABT and iABT enhance the formation of water molecules that can heal the disrupted colon by enhancing the gene expression of tight junction protein, Claudin 1 and ZO1 (Zonula Occludens). ABT modulates NFêB and Th17 pathways by inhibiting the expression of IL17 (Fig. 11).

The other therapy, it, regulates by up-regulating the anti-oxidative activity of superoxide dismutase (SOD). Upregulated anti-oxidative activities have healed the breached colonic epithelium by restoring the gene expression of Claudin 1. Restoration of colonic breaching has reduced the oxidative stress. This in turn have down-regulated the expression of pro-inflammatory cytokines (NFkB), which has inhibited the Th1 regulated gene expression (TNFα, IFNγ) (Fig. 11). Thus, it may be concluded from the above study that, novel combinatorial probiotics that are present in unstimulated and inulin stimulated whey water, possess significant therapeutic activities by regulating immune response.

Conflict of interest: Authors declare that there is no conflict of interest.

Author's contribution: SB: Involved in administration of dose regimen, performed the assays, standardization of α-amylase assay, written and edited the manuscript; AM: Involved in animal handling, written the abstract portion of this manuscript; ERB: Engaged in conceptualization, supervision and final editing of the manuscript.

Ethical approval: This *in vivo* experiment was performed according to the guidelines published by the Institutional and Departmental Animal Ethics Committee. Mice were housed under proper infection-free conditions at the departmental animal house. The ethical approval code is ERB/ZOO/2023/I, dated August 2nd, 2023.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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