Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 5 Issue 7 July 2022

Research Article

Novel 3-strain Combinatorial Probiotics Alleviates Symptoms of Inflammatory Bowel Disease in Mice

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DOI: 10.31080/ASMI.2022.05.1089

Received: May 06, 2022

Published: June 06, 2022

Abstract

Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal tract, with no permanent and safe cure. We designed this study, using a 3-strain novel combinatorial probiotic formulation (ABT) as therapy for IBD. The anti-inflammatory, pro-regenerative, and antibacterial activities of ABT were determined *in vitro*, and then validated in mice with IBD.

The anti-inflammatory activity of ABT on DSS-treated RAW cells was determined by MTT and NBT assays, and its antibacterial effect was determined against pathogenic bacteria. ABT was administered orally on days 5, 7, 9, 11 and 13, to mice with DSS-induced IBD. After sacrifice on day 14, disease parameters were measured.

ABT showed significant anti-inflammatory and pro-regenerative effects for over 96 hours, and inhibited 4 pathogenic bacterial strains. In mice, ABT successfully reduced symptoms of IBD, evidenced by restoration in body weight, cellular status, tissue structure, and mediators.

We inferred that the individual strains contributed cumulatively to the biological activities of ABT. The bacteria in the ABT adhered to the intestinal epithelium, and initiated repair mechanisms, by downregulating mediators (NFκB, TNFα, IL-1β, NO, and IAP), and upregulating mucus production. Thus, this translationally-valuable novel combination can be an economical and safe therapy for IBD.

Keywords: Combinatorial Probiotics; GRAS; Anti-inflammatory; Antibacterial; Adherence; Pro-regenerative

Abbreviations

AMPs: Antimicrobial Peptides; H_2O_2: Hydrogen Peroxide; sIgA: Secretory Immunoglobulin A; IBD: Inflammatory Bowel Disorder; GRAS: Generally Regarded as Safe; NFκB: Nuclear Factor Kappa B; IL8: Interleukin 8; TNFα: Tumour Necrosis Factor α; ABT: Novel Combinatorial Probiotics; WW: Whey Water; DMEM: Dulbecco's Modified Eagle Medium; DSS: Dextran Sulphate Sodium; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NBT: Nitroblue Tetrazolium; PBS; Phosphate Buffered Saline

Introduction

Probiotics, found in dairy food products, fermented foods, dietary supplements, and some non-dairy foods, are live, non-pathogenic microorganisms that, in sufficient amounts, benefit the health of the host [1-4]. Probiotic foods are beneficial food products containing probiotic micro-organisms in numbers sufficient [2]. Effects of probiotics are strain-specific [3]. Most common probiotics are bacteria from the genera *Lactobacillus* and *Bifidobacterium* [1,3-5]. Occasionally, strains of *Propionibacterium* and *Streptococcus*, and yeasts, like *Saccharomyces boulardii*, are also used [1,3-5].

Probiotic strains are not pathogenic to humans, are stable in physiological conditions, remain viable in the gastro-intestinal tract and intestine, can colonize the intestinal mucosa, and remain viable during packaging and storage [3-5].

Probiotics colonize the intestinal epithelium and inhibit the adherence of pathogens, through bacteriocins, antimicrobial peptides (AMPs) and hydrogen peroxide (H_2O_2), and/or regulate the body's immune system by producing immune-modulatory substances like secretory IgA (sIgA) and mucins, or by regulating phagocytosis and the production of cytokines and mediators, thereby restoring the body's normal microflora [1,2,5].

Over the years, probiotics have been used as supplementary therapy for several intestinal and non-intestinal diseases [3,5,6]. The US Food and Drug Administration has designated several probiotic strains as GRAS (Generally Recognized as Safe) since they are mostly non-toxic [3,6].

Several probiotic products are commercially available in India, from Amul, Mother Dairy, and Yakult [7]. However, these products mainly contain a strain of either *Lactobacillus* or *Bifidobacterium*, or a combination of the two, and are mainly used as dietary supplements. In this study, we have used a novel probiotic combination of three Gram positive strains- "A", "B", and "T". Due to confidentiality issues, the actual strains have not been disclosed.

The advantage of using these three particular strains, mixed in a proprietary ratio, lies in their different modes of action. While "A" and "B" are normal inhabitants of the gut, thus playing a role in restoring the lost microbiota in diseased conditions, "T" acts as a starter culture for "A". "A" produces bacteriocins, lactic acid and H_2O_2, which have antibacterial activities against pathogens [8]. It also shows anti-inflammatory effects, by inhibiting NFκB, Smad7, IL8 and TNFα, in gastric inflammation [8]. Stimulated by unmetabolized sugars and mucins in the colon, "B" enhances lactose digestion, stimulates host immunity, and improves intestinal barrier functions by increased production of mucus, AMPs, and sIgA [9,10]. Bile-intolerant "T" can survive transiently in the intestine, enhancing lactose digestion and modulating immune response [11,12]. "A" and "T" have a symbiotic relationship, where the casein-breakdown products of "A" stimulate "T", and the formic acid and carbon dioxide produced by "T" stimulate "A" [11-13].

In this study, we have validated the active period, the *in vitro* anti-inflammatory, antibacterial and adherence of this novel formulation.

Inflammatory bowel disorder (IBD), which is classified into ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disease of the gastrointestinal (GI) tract characterized by weight loss and diarrhea [14,15]. Both forms maybe caused by changes in the normal gut microflora, changes in the immune system, or due to genetic factors. While UC mainly affects the colon and rectum, and may cause rectal bleeding [15-17]. On the other hand, CD affects both the small and large intestines, as well as the mouth, esophagus, stomach and anus [15-17]. Although IBD, in general, is an autoimmune disorder caused by excessive immune response against intestinal microbiota, triggered either by hyperactivity of effector T cells or by reduced activity of regulatory T cells or by damage to the epithelial barrier, UC by itself elicits mainly a T_H^2 response, while CD elicits either a T_H^1 or a T_H^2 response [15,18].

IBD was initially considered a disease of the Western world, with the highest incidence in Northern Europe and Northern America, but with urbanization, industrialization, and their associated changes in environment, lifestyle and dietary habits, cases of IBD are rising rapidly in the East [19-21]. A shift of dietary pattern of the East to a Western diet, dominated by carbohydrates and red meat and deficient in fibres, is a major cause of IBD and loss of gut microbiota [20,21].

The etiology of IBD is not yet clear, though the involvement of the gut-associated microbiome and the mucus layer of the intestinal epithelium play significant roles. The intestinal epithelium, a single layer of intestinal epithelial cells (IECs), separated by tight junctions (TJs) and covered with mucus in which commensal bacteria are embedded, provides the first line of defense invading antigens [15,16]. Antigen recognition and processing, as well as innate and adaptive anti-microbial and anti-inflammatory responses, also begins at this layer [16]. These responses activate intracellular signaling pathways, resulting in the activation of NFκB and its downstream cytokine cascade, including activation of TNFα and IL1β, among other pro-inflammatory cytokines [15,16]. In IBD, the epithelial layer is damaged due to injury and subsequent dysregulation of TJ proteins, leading to reduction in epithelial

resistance and increase in permeability [16,22]. Impairment of antigen recognition and processing, changes in expression of TLRs and NOD1 stimulates the MyD88-dependent NFκB inflammatory cascade [16,22]. The balance between effector and regulatory T cells gets impaired, and production of mucosal barrier-associated molecules, like AMPs and mucin, get down-regulated [16,22].

Dextran sodium sulfate (DSS) is a water-soluble, negatively charged sulfated polysaccharide, is used to establish mouse models of IBD, usually for 4-10 days at concentrations of 2.5-5% [23]. It damages the TJ proteins, thereby disrupting the intestinal barrier functions, resulting in increase in permeability across the epithelium [23]. Mucin-production is significantly reduced, and there is increase in cellular infiltration and ulceration in the intestine and colon [23]. Cytokines like TNFα, IL1β, IL6, IL10, IL17 and TGFβ have been found to be involved in DSS-induced IBD [23].

The main challenges in IBD therapy, despite it being a common and high-incidence disease, is the lack of permanent and safe therapies, high expense of available therapy, and lack of medical insurance [19]. Apart from the high medical cost due to hospitalizations, surgeries, constant medical support, ambulatory care and pharmaceuticals, there are also indirect costs associated with unemployment, loss of productivity and reduced quality of life [19]. Currently, drugs for IBD include corticosteroids, salicylates, biologic agents and immune-modulators, which simply reduce the secondary symptoms of the disease without addressing the underlying mechanism, as a result of which, the rate of mortality in IBD patients still remain alarmingly high [24]. These drugs also are associated with adverse effects, and often do not get effectively delivered to the GI tract [24,25].

With these limitations in mind, we designed this study, where we have used a safe, natural and economic treatment for IBD, in the form of a probiotic formulation. In this study, the *in vitro* anti-inflammatory, pro-regenerative and antimicrobial activities of this novel combinatorial formulation, called ABT, comprising 3 strains- "A", "B" and "T" in a proprietary ratio, has been tested, followed by its validation in a mouse model of DSS-induced IBD.

Materials and Methods

Whey water preparation

The starter culture, named ABT, containing "A", "B" and "T" (obtained from Chr. Hansen Holding A/S, Denmark), was added to pasteurized, toned milk, boiled and cooled to 42-45˚C and incubated overnight at 42˚C, without agitation. After incubation, the whey water (labelled WW henceforth, and used for further assays) formed above the solid curd, was strained through a nylon mesh and collected in fresh tubes.

In vitro characterization

Anti-inflammatory and pro-regenerative effect over time

RAW 264.7 macrophage cells were cultured in DMEM. 10^4 cells were seeded into wells of a 96-well tissue culture plate (Tarsons, India). Two sets of adhered cells were treated with 3% DSS (SRL India) to induce inflammation. One set of DSS-treated cells was treated with 100 µl of WW, immediately after collection (0 hour), and every 8 hours, till 96 hours, and incubated overnight. In between each treatment, the WW was refrigerated at 4˚C. At the end of the overnight incubations, MTT and NBT assays were performed, and the proliferation and inhibition of superoxide radicals, respectively, were calculated [26-28].

Antibacterial activity

Antibacterial activity of WW (undiluted, 10^{-2} dilution, 10^{-4} dilution, and 10^{-6} dilution) against ten opportunistic and/or pathogenic bacteria of the gut (*Salmonella typhi, Vibrio cholera, Bacillus cereus, E. Coli DH5α, Vibrio parahaemolyticus, Enterococcus faecalis, Klebsiella sp., Staphylococcus aureus, Pseudomonas aeruginosa,* and *Staphylococcus epidermidis*) was assessed by the agar well diffusion method, and zones of inhibition, if any, were measured.

In vitro adhesion assay

Probiotics adhere to, and colonize, the intestinal epithelium, and secrete soluble factors to elicit the immune response [29]. An adhesion assay, using crystal violet, was done to determine whether WW or the individual components had any property of adhesion [30]. The adherence property of the sample (OD) is determined by its relation with blank medium (ODC), OD<ODC being non-adherent, ODC<OD<2ODC being weakly adherent, 2ODC<OD<4ODC being moderately adherent, and 4ODC<OD being strongly adherent.

In vivo validation

Ethical approval

All *in vivo* experiments have been performed according to rules laid down by the institutional and departmental animal ethics committee, and the animals were housed under specific pathogen free conditions at the departmental animal house.

Induction of disease and treatment with probiotics

Male BALB/c mice (6 weeks old, 20-25 gm) were divided into three groups (n = 4): Control, DSS, and DSS+Probiotics (D+P).

The mice of both the DSS and D+P groups were administered 3% DSS (in autoclaved water) orally, on days 0, 3, 5 and 7 [29]. Whey water (WW), prepared overnight, containing approximately 10^6 CFU of ABT, translating to a dose of 1.2 x 10^7 CFU/kg probiotics, was administered orally to the mice of the D+P group, on days 5, 7, 9, 11 and 13.

Measurement of body weight

The body weight of the mice of all 3 groups was recorded throughout the regimen.

Collection of tissues

Peripheral blood (PB) was collected by cardiac puncture, in K_2-EDTA tubes (BD Biosciences, USA). For DC count, smears were prepared on microscope slides. For serum, PB was collected in tubes, allowed to stand for at least 30 mins, centrifuged at 10000 rpm for 10 mins at 4˚C, the supernatant (serum) collected carefully in fresh tubes, and stored at -80˚C for later use.

Colon and small intestine, from each mouse, were cut into approximately 1 cm pieces for different assays. Pieces, cut into small digestible pieces, were incubated with a cocktail of 0.1% Collagenase I (Himedia, India) and 0.1% Collagenase IV (Himedia, India), in DMEM containing 1X Pen/Strep (Himedia, India), overnight at 37˚C. The cells were then filtered through a cell strainer to get a single cell suspension for TC count, CFU assay and immunophenotyping. For gene expression studies, tissues were stored in RNAlater solution (Ambion, Inc.) at -80˚C. Tissues were taken, washed with PBS and stored at -80˚C for protein expression studies. Tissues were collected in 10% buffered formalin for histological studies.

Total cell (TC) count

Cell suspensions were mixed with an equal volume of Trypan Blue dye (Himedia, India), and the cell viability was determined using a haemocytometer. The total number of viable cells per ml of cell suspension was calculated for each tissue.

Differential cell (DC) count

DC of leukocytes, key players in inflammation, is an indication of the type or stage of the diseases. Blood smears were air dried, fixed with methanol (SRL, India), stained with Giemsa stain (SRL, India) for 15 mins, washed and observed under a light microscope (Dewinter Fluorex LED) at 40X magnification. The percentage of each cell type, distinguished on the basis of their nuclear morphology, was calculated.

Determination of immune cell migration by immunophenotyping

Immunophenotyping was done to determine the effect of WW on the migration of each immune cell subset. Cell surface staining was performed on PB and colon cells using the following antibodies: hematopoietic cell marker CD45-PerCP/Cy5.5 (BioLegend), T cell marker CD3e-PE (BD Biosciences, USA), helper T (T_H) cell marker CD4-V450 (BD Biosciences, USA), cytotoxic T (T_C) cell marker CD8a-AF488 (BD Biosciences, USA), B cell marker B220-FITC (BD Biosciences, USA), neutrophil marker GR-1-FITC (MACS), and macrophage/monocyte marker F4/80-PE (Invitrogen). Flow cytometry was done on BD Accuri (BD Biosciences, USA), and the number of each cell subtype was determined from the TC count of that tissue.

Determination of nitric oxide content by Griess reaction

The concentration of serum nitric oxide (NO) was estimated using Griess reagent, with a standard curve prepared using sodium nitrate (SRL, India).

Alkaline phosphatase activity

Alkaline phosphatase (AP) is a heat-stable enzyme, active at alkaline pH, which dephosphorylates compounds. It is present in many parts of the body, and acts as a marker of inflammation [31]. Serum AP (ALP) increases and intestinal AP (IAP) decreases in IBD. Activities of ALP and IAP were assessed, using a protocol modified from that used by Kanta., *et al.* [32], which uses the ability of AP to dephosphorylate p-nitrophenol phosphate (PNPP), to

p-nitrophenol (PNP). The activity of ALP and IAP {in µmol/min, equivalent to International Units (IU)/litre} was calculated using the formula: ALP activity (µmol/min=IU/L) = Amount of PNP (from standard curve)/15.

Gene expression by reverse transcriptase PCR (RT-PCR)

Since the DSS-induced IBD model has characteristics of both T_H^1 and T_H^2 responses, expression of genes involved in both pathways, like iNOS, TNFα, IL-4, IL-13, IL-1β, TGFβ, and IFNγ were assessed by RT-PCR, using manufacturers' protocol (Trizol reagent and Verso cDNA synthesis kit, Life Technologies, USA), with GAPDH as the housekeeping gene. PCR products were run in 1% agarose gel (G-Biosciences, USA), with a 100 bp marker (100-2000 bp; GeNet Bio, Korea); bands were observed under UV light in a gel-doc system (BioRad), and the intensities of the bands were determined using ImageJ.

Protein expression by western blot

The expressions, at the protein level, of the transcription factors and signaling molecules, like NFκB, NOS_2, arginase, and HIF1α, expressed in the colon, was measured by western blot, using GAPDH as the housekeeping protein. Total protein was extracted from the colon using RIPA buffer, estimated by Bradford reagent, resolved on a 12% SDS-PAGE, and western blot performed, using rabbit anti-mouse GAPDH (G Bioscience), rabbit anti-mouse NFκB (G Bioscience), rabbit anti-mouse HIF1α (Santa Cruz), rabbit anti-mouse NOS_2 (Santa Cruz), rabbit anti-mouse arginase (Santa Cruz) primary antibodies, and goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz). The blot was developed using Western ECL substrate (BioRad), visualized using a chemidoc system (BioRad), and the band intensities were determined using ImageJ.

Histology

Colon and intestine tissues were fixed with formalin, dehydrated, embedded in paraffin and cut into 5 µm sections using a rotary microtome. The sections were stained with hematoxylin and eosin (to assess cellular infiltration) and alcian blue (to assess mucus-containing Goblet cells). The stained sections were observed under a light microscope at 10X (Dewinter Fluorex LED) and 20X (Olympus BX41) magnifications.

Colony forming unit-cell (CFU-c) assay

The clonogenic potential (ability of the cells to grow and proliferate into colonies) of blood, colon and intestinal cells was assessed, using methyl-cellulose semisolid media, in the presence of a stem cell stimulating factor. The clonogenic potentials of the samples were calculated by dividing the number of colonies formed after 7 days by the total number of cells plated.

Statistical analysis

All data (represented as Mean ± SEM) were analyzed, and statistical significance ($p < 0.05$) calculated by t- test, using GraphPad Prism 6.

For the *in vitro* studies, * has been used to denote significance with respect to untreated RAW 264.7 cells, and # has been used to denote significance in comparison to DSS-treated RAW 264.7 cells. In the adhesion assay, * has been used to denote significance with respect to WW.

In the *in vivo* assays, * has been used to denote significance with respect to control samples, and # has been used to denote significance in comparison to DSS-treated samples.

Results, Discussion and Conclusion

Inflammatory bowel disorder is a chronic, debilitating, inflammatory disease of the gastrointestinal tract, characterized by weight loss and diarrhea, often with rectal bleeding [14,15]. Ulcerative colitis and Crohn's disease, the two types of IBD, may be caused by invasion of pathogens, changes in the immune response, changes in the normal gut microbiota, environmental factors, and genetic factors, mostly in conjunction with each other [14,15]. Initially IBD was more prevalent in the western world, mainly due to their dietary habits of high protein and low fibres [19-21]. However, changes in the lifestyle and dietary practices of the eastern world have now led to a rapid rise in IBD cases in the east as well [19-21].

Gut microbiota play a critical role in the development of IBD. Changes in the microbiota, either due to colonization by other pathogenic organisms, or by some other factor, lead to imbalance in the intestinal homeostasis. One of the main effects of IBD is a leaky epithelial barrier that allows the entry of more antigens, leading to the expression of pro-inflammatory cytokines and their subsequent signaling cascades. Current therapy for IBD, which includes corticosteroids, salicylates, biological agents (like TNFα inhibitors), and immune-modulators, do not address the underlying mechanisms completely, relying more on simply

reducing the symptoms and maintaining patients in remission [24,25]. These drugs also have side effects, causing the disease progression and mortality to still remain significantly high [24,25]. Diet therapy is also used for IBD, but none can completely cure the disease, leading to high costs incurred by the patient to for continuous medical care [25]. Another staggering limitation of IBD therapy are the extremely high direct cost of treatment, as well as indirect costs, associated with unemployment, loss in productivity and reduced quality of life [19,24].

In this study, we have used a novel combination of probiotic bacteria as an alternative treatment strategy for IBD.

Probiotic therapy involves the administration of live, non-pathogenic organisms, mainly bacteria, via food (mostly fermented foods and dairy products), with the aim of benefitting the health of the individual. It attempts to restore, or stimulate, the commensal bacteria of the gut and activate repair mechanisms of the body. Multi-strain probiotics appear to be more effective than single-strain probiotics, though most studies have used a combination of only 2 strains. Most formulations contain only one or two strains [4,33,34]. While each strain, by themselves, have health benefits, our formulation has an advantage over existing formulations in that it is made of 3 beneficial strains. Thus, it may be safe to say that the combination of 3 strains in our formulation will also be beneficial.

Probiotics compete with, and prevent colonization of, pathogens on the intestinal epithelium, and regulate immune response, by acting on the intestinal tight junctions, and sealing leaky barriers [3,4,29]. They release soluble factors that act directly on the inflammatory pathways, and stimulate the release of mediators [3,4,29].

In this study, we have performed *in vitro* characterization of our novel combination, to determine its possible mechanism of action, followed by its validation in an *in vivo* mouse model of IBD.

The novel combinatorial probiotic, ABT, was inoculated in boiled and cooled milk, to obtain whey water, which was used for further studies. Having determined the growth period of the WW and its individual strains, *in vitro* anti-inflammatory, pro-regenerative and antibacterial activities were assessed.

The active period of WW was found to stretch over 96 hours, which we found to be a cumulative effect of the growth periods of the individual strains. This was corroborated by the MTT and NBT assays, where we found that, even after 96 hours, the WW was successful in restoring the proliferation of the cells (Figure 1A), and scavenging superoxide radicals (Figure 1B, 1C), respectively. As mentioned earlier, the WW was stored at 4˚C before administering to the DSS-treated cells. Thus, this experiment not only shows the anti-inflammatory and proliferative effects of the WW, it also gives us an idea about its refrigerated shelf life. While the activity decreases slightly from approximately 80 hours to 96 hours, it is also clear that there is still significant anti-inflammatory and proliferative effects even at that time.

In the MTT assay, we found that, the proliferation of all the cells, grown with no addition of fresh media, decreased with time. The proliferation of the DSS-treated cells (Figure 1A, red) was significantly lower than the untreated cells (Figure 1A, blue), which was significantly restored by treatment with WW (Figure 1A, green). This shows that WW has the ability to stimulate the growth of inflamed cells.

In the NBT assay, we found that the release of O_2^- by the untreated RAW cells was minimal (Figure 1B, blue), whereas that by the DSS-treated cells was significantly higher (Figure 1B, red) throughout the duration of the experiment. This was significantly brought down by WW (Figure 1B, green), although the production of O_2^- by the DSS-treated cells treated with WW rose as time passed. As a result, it was evident that the anti-inflammatory activity and the O_2^--scavenging activity of the WW (Figure 1C), though higher at the earlier stages of the experiment, remained significant even after 96 hours.

We found that WW has significant antibacterial effect against several pathogenic strains of bacteria, including *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus cereus*, and *Vibrio parahaemolyticus* (Table 1A). All four pathogens, which mainly affect the GI tract, were inhibited most by the undiluted WW. Earlier studies have shown that "A" and "B" show anti-bacterial effect against several other strains of bacteria, by producing bacteriocins and H_2O_2, while "T" shows limited antibacterial effect [8-13]. In the combination, it is possible, "A" provides the major antibacterial effect against these test bacteria, while the other two strains may also produce some bactericidal factors.

Figure 1: [A] The proliferation of DSS-treated RAW 264.7 cells was restored significantly ($p < 0.05$), throughout the duration of 96 hours, with the administration of WW. [B] The superoxide generation by DSS-treated RAW 264.7 cells was inhibited significantly ($p < 0.05$), throughout 96 hours, with the administration of WW. [C] The superoxide radical-scavenging activity of WW, though present even at 96 hours, decreased with time. (*: $p < 0.05$, compared to untreated RAW 264.7 cells; #: $p < 0.05$, compared to DSS-treated RAW 264.7 cells).

a	**Inhibition Zone Diameters (cm)**			
	WW	**WW- 10^{-2}**	**WW- 10^{-4}**	**WW- 10^{-6}**
S. epidermidis	1.6 ± 0.07	1.0 ± 0.03	0.9 ± 0.03	0.7 ± 0.06
E. faecalis	1.1 ± 0.03	1.1 ± 0.03	1.0 ± 0.06	0.9 ± 0.06
B. cereus	1.1 ± 0.06	0.8 ± 0.03	0.3 ± 0.07	0.0 ± 0.00
V. parahaemolyticus	1.6 ± 0.06	1.4 ± 0.10	1.3 ± 0.06	1.2 ± 0.06
Klebsiella	No Inhibition	No Inhibition	No Inhibition	No Inhibition
S. typhi	No Inhibition	No Inhibition	No Inhibition	No Inhibition
V. cholerae	No Inhibition	No Inhibition	No Inhibition	No Inhibition
S. aureus	No Inhibition	No Inhibition	No Inhibition	No Inhibition
P. aeruginosa	No Inhibition	No Inhibition	No Inhibition	No Inhibition
E. coli DH5α	No Inhibition	No Inhibition	No Inhibition	No Inhibition
b	**OD/ODC**	**Adherence**		**Fold change, with respect to WW**
WW	3.04 ± 0.06	Moderately adherent		
A	2.39 ± 0.03	Moderately adherent		(-) 1.27*
B	2.29 ± 0.07	Moderately adherent		(-) 1.33*
T	2.39 ± 0.12	Moderately adherent		(-) 1.27*

Table 1: a: Growth of bacterial strains, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus cereus* and *Vibrio parahaemolyticus*, was inhibited by whey water. Inhibition was inversely proportional to dilutions of WW. WW did not have any inhibitory effect on *Klebsiella sp*., *S. typhi*, *V. cholera*, *S. aureus*, *P. aeruginosa*, and *E. coli* DH5α.

b: Whey water, and all three components, were moderately adherent to the surface. (*: $p < 0.05$, compared to WW combination).

To determine whether ABT could adhere to any surface, we performed the crystal violet assay. We found that WW showed significantly better adherence than the individual strains, indicating a possible mechanism of action for the combination (Table 1B).

Thus, from the *in vitro* results, we could determine that ABT had an active period of 96 hours, had significant anti-inflammatory and pro-regenerative effects, showed antibacterial activity against some pathogenic strains, and also showed the property of adherence. The long active period of WW allows for a significantly long shelf-life. From the above observations, and from available information about the three component strains of our combination, we hypothesized that the "T" acts as a starter, stimulating the growth of the other two strains and activating their immune-modulatory pathways. The ability of WW to adhere to surfaces may allow it to adhere to the intestinal epithelium and initiate anti-inflammatory mechanisms, when administered *in vivo*.

With these *in vitro* results, we administered the WW to DSS-treated mice, to determine its *in vivo* effect. WW administration was started on day 5, once the disease had already set in.

Weight loss is one of the most prominent presentations of IBD, often seen even before diagnosis of the disease [35]. Patients often develop an aversion to eating, because they associate eating with gastrocolic reflex and abdominal pain [35]. Additionally, the inflammatory nature of the disease, and the energy expended during the acute flare-ups lead to a catabolic and anorexic effect [35]. Expression and production of metabolic hormones and absorption of macro- and micronutrients are also affected [35]. We found that administration of WW led to a significant restoration of the body weight of the mice, which was lost due to DSS treatment (Figure 2).

Figure 2: Whey water restored the body weight by 1.23 fold ($p < 0.05$) compared to the DSS group on day 14. (*: $p < 0.05$, compared to control; #: $p < 0.05$, compared to DSS).

Cellular infiltration to the tissue of interest, through the blood, is a key marker of inflammation. WW significantly reduced the infiltration of cells, as well as the number of $CD45^+B220^+$ B cells, $CD45^+CD3^+$ T cells, $CD45^+CD3^+CD4^+$ T_H cells, $CD45^+CD3^+CD8^+$ T_C cells, $CD45^+GR1^+$ neutrophils, and $CD45^+F4/80^+$ macrophages/monocytes in the blood, which as seen by both the Giemsa staining (Figure 3A) and flow cytometry (Figure 3C, 3D, 3E). IBD severity has been found to be directly proportional to the neutrophil-to-lymphocyte ratio (NLR) and inversely proportional to the lymphocyte-to-monocyte ratio (LMR) [36]. The NLR and LMR were also restored significantly with WW-treatment, indicating a reduction in the inflammation (Figure 3B).

Figure 3: [A] Whey water inhibited the infiltration of total cells, neutrophils, lymphocytes and monocytes in the blood, respectively, by 1.57 fold ($p < 0.05$), 2.27 fold ($p < 0.05$), 1.07 fold, and 1.89 fold ($p < 0.05$). [B] WW restored the NLR and LMR in the blood by 2.12 fold ($p < 0.05$), and 1.77 fold ($p < 0.05$), respectively. WW reduced the B220+ B cells [C] by 1.63 fold ($p < 0.05$), the CD3+ T cells [D] by 2.37 fold ($p < 0.05$), the CD4+ TH cells [D] by 1.19 fold, the CD8+ TC cells [D] by 3.57 fold ($p < 0.05$), the GR1+ neutrophils [E] by 1.06 fold, and the F4/80+ macrophages and monocytes [E] by 11.71 fold ($p < 0.05$). [F] The clonogenic potential of blood was restored by 1.97 fold ($p < 0.05$) in the D+P group. [G] WW reduced the activity of serum alkaline phosphatase by 1.69 fold ($p < 0.05$). [H] WW inhibited the production of serum nitric oxide by 2.04 fold ($p < 0.05$). (*: $p < 0.05$, compared to control; #: $p < 0.05$, compared to DSS).

In the colon and intestine, it has been seen that IBD leads to a higher proportion of memory B cells, activated $CD4^+$ T cells, follicular helper T cells, M1 macrophages, dendritic cells (activated & resting), activated mast cells, and polymorphonuclear leukocytes [37]. The counts of $CD45^+B220^+$ B cells, $CD45^+CD3^+$ T cells, $CD45^+CD3^+CD4^+$ T_H cells, $CD45^+CD3^+CD8^+$ T_C cells, $CD45^+GR1^+$ neutrophils, and $CD45^+F4/80^+$ macrophages/monocytes in the colon were increased with DSS, and significantly inhibited by WW-administration (Figure 4A, 4E-G). The architecture, especially the epithelial layer, of the colon, which was damaged by DSS, was also successfully restored by WW, as seen by H & E staining (Figure 4B-D). Cellular infiltration was also inhibited in the intestine, and the structure also repaired, by WW (Figure 6A-D).

Figure 4: [A] The total cellular infiltration in the colon was inhibited by 1.43 fold ($p < 0.05$) with WW treatment. Hematoxylin-eosin staining of colon cross-sections, observed under light microscope (Dewinter Optical Inc., India), at 4X magnification, showed increased cellular infiltration, and damaged epithelium in the DSS-treated colons [C] compared to control [B], which decreased with administration of WW [D]. WW reduced the B220+ B cells [E] by 1.40 fold ($p < 0.05$), the CD3+ T cells [F] by 2.80 fold ($p < 0.05$), the CD4+ TH cells [F] by 4.18 fold ($p < 0.05$), the CD8+ TC cells [F] by 2.00 fold ($p < 0.05$), the GR1+ neutrophils [G] by 1.56 fold ($p < 0.05$), and the F4/80+ macrophages and monocytes [G] by 1.02 fold. [H] The clonogenic potential of colon was restored by 1.60 fold ($p < 0.05$) in the D+P group. (*: $p < 0.05$, compared to control; #: $p < 0.05$, compared to DSS. Scale bar for B-D: 100 μm).

Figure 5: Alcian blue staining of cross-sections of colon, observed under light microscope (Olympus BX41), at 20X magnification, showed reduced Goblet cells (red arrows in B) in the DSS-treated colons [B] compared to control [A], which increased with administration of WW [C]. [D] Gene expressions of TNFα, IL1β, IL-4 and TGFβ respectively decreased by 1.60 fold ($p < 0.05$), 1.20 fold ($p < 0.05$), 1.22 fold ($p < 0.05$), and 1.09 fold ($p < 0.05$) with WW treatment. [E] Gene expressions of IFNγ, IL-13 and iNOS respectively decreased by 1.18 fold ($p < 0.05$), 1.79 fold ($p < 0.05$), and 1.22 fold ($p < 0.05$) with WW treatment, with GAPDH remaining the same. [F] Protein expressions of NFκB, NOS2, and HIF1α respectively decreased by 2.15 fold ($p < 0.05$), 3.64 fold ($p < 0.05$), and 4.26 fold ($p < 0.05$), the expression of arginase increased by 7.20 fold ($p < 0.05$), with WW treatment, while expression of GAPDH remained same. (*: $p < 0.05$, compared to control; #: $p < 0.05$, compared to DSS. Scale bar for A-C: 100 μm).

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Release of inflammatory mediators led to tissue degeneration, and subsequent loss in clonogenic potential, which was restored by WW, in the blood and colon (Figure 3F, 4H). This implies that the WW also exerts a regenerative effect on the tissues.

The mucus layer along the GI tract, formed by mucin secreted by Goblet cells, is most important for mucosal defense [22,38]. Normally, mucin granules are present throughout the colon and intestine. When stained with Alcian blue, the stain is confined to the mucin granules in the Goblet cells, with little to no staining of perinuclear cytoplasm [38]. On the other hand, it has been seen that, in IBD, the number of Goblet cells were significantly reduced, as were the mucin granules, and the perinuclear cytoplasm was expanded and prominently stained [38]. In our sections of colon and intestine, as well, we saw a restoration in the number of mucus-producing Goblet cells, after WW-treatment (Figure 5A-C, 6F-H).

In inflammation, the total serum alkaline phosphatase increases, acting as a marker of inflammation [31]. On the other hand, the intestinal AP, expressed by the brush border of the intestinal epithelium, decreases in IBD [31]. This is most likely due to the extensive damage to the intestinal epithelial layer, leading to the loss in the brush border cells, and reduced expression of IAP. Since IAP is active in dephosphorylating pro-inflammatory molecules, like bacterial LPS and flagellin, as well as ATP, and in preventing activation of NFκB, via MyD88-dependent pathway, its decreased production in IBD becomes extremely important in the progression of the disease [31]. WW was successful in restoring the levels of serum AP and IAP significantly (Figure 3G, 6E).

Serum NO, another important mediator of inflammation, has been found to increase in IBD, as do NO in the lumen of the colon, in the intestinal mucosa and in the rectum [39]. This over-production in mucosal inflammation is driven by the over-expression of iNOS, the enzyme responsible for producing NO from L-arginine [39]. WW successfully reduced the level of serum NO (Figure 3H), as well as the expression of iNOS in the colon (Figure 5D, 5F).

Analysis of gene and protein expression showed that WW down-regulated the expression of the pro-inflammatory cytokines in the colon, TNFα, IL1β, IL-4 and TGFβ, as well as the downstream mediators which are activated by these cytokines, including IL-13, iNOS and IFNγ (Figure 5D-F). Arginase competes with iNOS for the common substrate L-arginine, where arginase converts it to ornithine and urea, and iNOS converts it to nitric oxide and L-citrulline [40]. This inverse relationship between iNOS and arginine is confirmed by our Western blot, where we found that arginase is down-regulated with DSS-treatment, and up-regulated again with WW-treatment, going opposite to the trend of iNOS (Figure 5F). As mentioned before, the role of NFκB is well-

documented in IBD. Our observations showed that the expression of NFκB was also inhibited by WW, which would, in turn, impact the expression of its downstream products (Figure 5F). IBD also leads to increased hypoxia in the intestinal mucosa, associated with the transcription factor HIF1α, which increases barrier protective genes, elicits the innate immune response and activates anti-microbial response and HIF2α [41]. Our observations show the over-expression of HIF1α with DSS-treatment, indicating the increase in hypoxia in the tissue, and its role in the immune response mounted against DSS, which was reduced by WW (Figure 5F).

From all the above observations, and from what is known about the three strains of bacteria present in our probiotic combination, we have hypothesized how the ABT probiotics work. "T" acts as a starter for the other two strains, stimulating their growth. While "A" and "B" adhere to the epithelial layer, as seen by the adhesion assay, "T" may not necessarily adhere. However, all three strains have been known to be able to stabilize junctional proteins, thereby repairing the leaky junctions [42]. This ability of WW to repair the leaky epithelium has been studied earlier in our lab, where we co-cultured RAW 264.7 macrophage cells (to simulate the immune cells) with Caco2 cells (to simulate the intestinal epithelial layer), to assess the trans-epithelial resistance (TER) between the two [29]. We found that administration of WW significantly increased the TER, indicating its ability to repair leaky epithelial layers [29]. The three probiotic strains in WW compete with the pathogenic bacteria for adhesion sites on the epithelium, and probably displace the pathogens or even kill them by secreting antibacterial peptides or bacteriocins. "T" is able to promote the growth of "A" by releasing formic acid and carbon dioxide. "A" can inhibit NFκB, TNFα and IL-8, thereby inhibiting their downstream functions, cytokines and mediators, leading to the reduction in T_H^1 and T_H^2 responses, neutrophil recruitment, as well as release of mediators like NO. "B" also promotes the production of mucus, thus repairing the damaged mucus layer protecting the epithelium. The repaired epithelial cells, in turn, regain their ability to produce enzymes like alkaline phosphate, which in turn, help regulate the gut bacteria, as mentioned earlier. Ultimately, the epithelial layer, as well as any other damage to the tissue, gets repaired, by restoration of the normal healing mechanisms.

The mechanism by which IBD progresses and the probable mechanism by which whey water acts in treating it have been outlined in figure 7A and figure 7B respectively.

Figure 7: [A] Diagrammatic representation of the series of events that drive the progression of inflammatory bowel disease. IBD leads to overgrowth of pathogens, depletion of commensals, reduction in Goblet cells and mucus, depletion of the protective mucus layer, damaged junctional proteins, increased permeability of the epithelium, reduced alkaline phosphatase, increased of influx of antigens, increased NFκB cascade, increased recruitment of leukocytes, increased TH1 and TH2 responses, and increased mediators (NO, ROS, histamines), ultimately leading to tissue damage. The red arrows in the figure indicate the upregulation or down-regulation of the cytokines and mediators involved in IBD. Maroon arrows indicate the influx of pathogens, antigens, and other inflammatory molecules. [B] Our observations led us to infer that the ABT most likely competes with pathogens (red curved arrow) and colonizes the epithelium, signaling the repair of the leaky barrier by stabilizing the junctional proteins, up-regulation in the production of mucus, up-regulation in the production of dephosphorylating enzymes, and also the down-regulation of inflammatory genes in the mucosa. This, in turn, leads to the reduction of inflammatory mediators, and ultimately to repair of the damaged tissue. The red arrows in the figure indicate the points at which the three bacterial strains in our mixture act directly, while the yellow arrows indicate the downstream effect of their actions. Yellow crosses indicate the points where the ABT brings about inhibition.

From the above observations, we can conclude that this novel probiotic combination is successful in reducing IBD in mice. It not only reduces the secondary symptoms, like most existing drugs, but also attacks the disease in its mechanistic pathways. It is also hassle-free in preparation and administration, being given orally every other day. Being a functional food, it also does not have the harmful side effects, characteristic of other drugs and biologics. Preparation of the whey water to obtain the active ABT is rapid and economical. Compared to other commercially available probiotic formulations, which have one or two strains, this combination works through the combined effect of 3 strains, each of which exert their effects in an overlapping manner. Therefore, this novel combination, if translated for clinical use, may well be an economical and safe therapy for IBD.

Acknowledgement

We would like to acknowledge the support of Dr. Sukhendu Mandal (Assistant Professor, Department of Microbiology, University of Calcutta) for providing us with the panel of pathogenic bacterial strains used in the antimicrobial assay. We would like to acknowledge the University Grants Commission (UGC) the Indian Council of Medical Research (ICMR) for providing grants to ERB, and to UGC for providing fellowship for SM.

Conflict of Interest

The authors declare that there is no conflict of interest or financial interest.

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