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ORIGINAL CONTRIBUTION

Active hexose-correlated compound and *Bifidobacterium longum* BB536 exert symbiotic effects in experimental colitis

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Abstract
Purpose Active hexose-correlated compound (AHCC) is a commercial extract obtained from Basidiomycetes under controlled conditions, yielding a 74 % content in oligosaccharides, especially α-glucans. AHCC has a number of therapeutic effects, including intestinal anti-inflammatory activity. *Bifidobacterium longum* BB536 is a probiotic with potential health-promoting effect at the gut level. The purpose of the present study was to evaluate the possibility of synergism between AHCC, which is believed to act as a prebiotic, and *B. longum* BB536.
Methods We used the trinitrobenzene sulfonic acid model (TNBS) of colitis in rats. AHCC (100 or 500 mg kg^{-1}) and *B. longum* BB536 (5×10^6 CFU rat^{-1} day^{-1}) were administered together or separately for 7 days prior to colitis induction and then for another 7 days and compared with control (noncolitic) and TNBS rats.
Results The results show that both treatments had intestinal anti-inflammatory activity separately, which was enhanced when used in combination, as shown by changes in body weight gain, colonic weight to length ratio, myeloperoxydase activity and iNOS expression. Interestingly, the association of AHCC 100 mg kg^{-1} + *B. longum* BB536 showed the highest anti-inflammatory activity.
Conclusions Our data provide a preclinical experimental basis for the synergistic effect of AHCC and *B. longum* BB536 on inflammatory bowel disease.

Keywords Active hexose-correlated compound · *Bifidobacterium longum* BB536 · Symbiotic · Colitis

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Introduction

Inflammation of the intestine/colon is an important feature of gastrointestinal pathology, especially in chronic inflammatory conditions of the gut, known as inflammatory bowel disease (IBD). IBD comprises essentially Crohn's disease and ulcerative colitis. These are idiopathic and recurring inflammatory conditions that affect greatly the patients' life, due to the need for close monitoring, surgery, and interference with day-to-day activities. IBD is incurable to date, except for the ulcerative colitis patients undergoing total colectomy. However, IBD patients can be successfully managed in most cases with the use of intestinal anti-inflammatory drugs, including corticoids and immunosuppresants, aminosalicylates, and anti-TNF molecules. None of these therapies targets the (unidentified) causes of IBD and act in an unspecific fashion. Not surprisingly, treatment must frequently deal with resistance and severe side effects. Hence, there is a well-recognized need for new therapeutic alternatives [1].

Because of its continuity with the external surfaces of the body, the gastrointestinal tract is colonized by bacteria and other microorganisms. However, it is a unique organ system in the human body because there is no physiological provision for containment at any given section, and therefore, the entire tract contains a gastrointestinal flora or

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microbiota, which is most prominent in the terminal ileum, cecum, and colon. In fact, the bacterial population outnumbers the eukaryotic cells not only in the digestive system, but in the human body as a whole [2]. Although not essential for life, the intestinal flora is in symbiosis with the host, providing butyrate for colonocytes as an important nutrient, digesting carbohydrates otherwise unusable by the host, and acting as a buffer against noxious invading microorganisms. While the interplay between the different bacterial species present in the gut and the intestinal mucosa is far from being well characterized, it is generally accepted that some bacteria have beneficial effects on the host, as opposed to potentially harmful species such as Clostridia or *Escherichia coli*. In other words, the equilibrium in the intestinal flora appears to be a fundament of gut homeostasis. The 'host-friendly' bacteria pushing in one side of this equilibrium include *Saccharomyces boulardii*, *E. coli* Nissle 1917, *Lactobacillus,* and *Bifidobacterium* species, and are known generically as probiotics. Probiotics have been claimed to combat infectious diarrhea, specially traveler's diarrhea, irritable bowel syndrome and metabolic syndrome [3].

The other means of accomplishing a correct balance in the gut flora is through the use of prebiotics [4]. These are nondigestible, nonabsorbable oligosaccharides that reach the distal sites of the gastrointestinal tract unchanged and are used by residing bacteria as nutrients. Although the exact mechanisms are not clear, prebiotics somehow favor the selective growth of beneficial bacteria, thereby achieving the same goals as probiotics, at least in theory. Therefore, pre- and probiotics are expected to exert symbiotic effects by modulating the flora via different mechanisms. Active hexose-correlated compound (AHCC) is a commercial extract obtained from Basidiomycetes under controlled conditions, yielding a 74 % content in oligosaccharides, specially α-glucans [5]. We have previously established that AHCC is beneficial in a preclinical IBD model and that its mode of action is consistent with a prebiotic mechanism [6]. In this study, we aim to enhance AHCC effects by adding a probiotic, namely *Bifidobacterium longum* BB536.

Materials and methods

Except where indicated all reagents were purchased from Sigma (Madrid, Spain). AHCC was provided by Amino Up Chemical (Sapporo, Japan), whereas *B. longum* BB536 was supplied by Morinaga (Tokyo, Japan).

Animals

Female Wistar rats (200–250 g) obtained from Janvier (Le Genest Saint Isle, France) were used, housed in macrolon cages, and maintained in air-conditioned animal quarters with a 12-h light/dark cycle. Rats were provided free access to sterilized tap water and standard rodent chow. Diet composition (Harlan Teklad Global Diets, Castellar, Spain) was (wt:wt) 15.4 % protein, 2.9 % fat, 60 % carbohydrates, 3.9 % fiber, 5.3 % minerals, and 12 % moisture. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and was approved by the Animal Welfare Committee of the University of Granada (Granada, Spain).

Induction of colitis

Briefly, rats were fasted overnight and anaesthetized with halothane. Under these conditions, rats were given 10 mg of trinitrobenzene sulfonic acid (TNBS) dissolved in 0.25 mL of 50 % ethanol (v:v) by means of a Teflon cannula inserted 8 cm through the anus. The mechanism of TNBS-induced colitis involves reaction of the hapten with host tissue proteins, generating a variety of new antigens in situ as well as the production of free radicals. Rats were kept in a head-down position for an additional 30 s and returned to their cage [7].

Experimental design

Rats were randomly assigned to 7 different groups ($n = 5$ except $n = 4$ or 6 for the Control and TNBS groups, respectively). For ethical reasons, group size was designed to be as small as possible based on a power analysis estimated from previous similar experiments from our group. Six groups (TNBS, AHCC 100, AHCC 500, Probiotic, AHCC 100 + probiotic, and AHCC 500 + probiotic) received the TNBS challenge to induce colitis as described above, whereas the Control group was given 0.25 mL of PBS intrarectally. Groups AHCC 100 and AHCC 100 + probiotic received 100 mg kg^{-1} day^{-1} of AHCC in distilled water orally, whereas AHCC 500 and AHCC 500 + probiotic were given 500 mg kg^{-1} day^{-1} by the same procedure. Irrespective of AHCC administration, the probiotic, AHCC 100 + probiotic and AHCC 500 + probiotic groups received 5×10^6 CFU rat^{-1} day^{-1} in distilled water, keeping a 2-h gap from the previous AHCC oral gavage in order to avoid direct probiotic–prebiotic interactions. Rats from Control and TNBS groups received the corresponding vehicle daily.

Rats received the prebiotic and/or probiotic for 7 days before TNBS challenge and also 7 days thereafter until killed. An esophageal catheter was used to deliver all treatments. Food intake, water intake, and body weight were measured every day.

Assessment of colonic damage

Rats were killed by cervical dislocation, and the entire colon was removed, placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed, and its length measured under a constant load (2 g). The large intestine was longitudinally opened and scored for visible damage on a 0–25 scale according to the following criteria: adhesions (0–3), obstruction (0–2), thickening (0–2), hyperemia (0–3), fibrosis (0–3), necrosis (0–5), and scarring and deformation (0–7). The colon was subsequently divided longitudinally in several pieces for biochemical determinations. One of these pieces was used for Western Blot assays. The other fragments were immediately frozen in liquid nitrogen and kept at −80 °C until used. A single piece of colon was homogenized per animal in order to measure myeloperoxidase (MPO) and alkaline phosphatase (AP) activities. MPO activity was determined according to the technique described by Krawisz et al., using 0.5 % hexadecyltrimethylammonium bromide in Tris (pH = 6.0) for tissue homogenization and o-dianisidine dihydrochloride (533 mmol L^{-1}) as chromogen [8]. AP activity was measured spectrophotometrically, using disodium nitrophenylphosphate (5.5 mmol L^{-1}) as substrate in 50 mmol L^{-1} glycine buffer with 0.5 mmol L^{-1} $MgCl_2$ (pH = 10.5). Enzimatic activities are expressed as mU mg $protein^{-1}$ [9].

The percentage of necrosis along the entire large intestine surface was assessed making use of ImageJ computer software (National Institutes of Health). The relative quantification was carried out on high-resolution pictures taken immediately before the colon was split into different samples.

The spleen was removed, weighed, snap frozen in liquid nitrogen and kept at −80 °C. Mesenteric lymph nodes were removed and cleaned of fat and mesentery, and then mechanically disrupted with forceps in FBS-free DMEM to release cells. After centrifugation (1,500 rpm, 4 °C, 5 min), the pellet was resuspended in RPMI supplemented with 10 % (v/v) FBS, glutamine (2 mmol L^{-1}), penicillin (100 kU L^{-1}), streptomycin (0.1 g L^{-1}), amphotericin B (2.5 mg L^{-1}), and 2-mercaptoethanol (0.05 mM). Cells were cultured at a density of 0.5×10^6 cells $well^{-1}$ with or without concanavalin A (5 μg mL^{-1}), for 48 h at 37 °C under standard culture conditions. Cell culture medium was collected after 48 h, cleared by centrifugation (10,000 rpm, 4 °C, 5 min), and frozen at −80 °C until assayed for cytokine content using commercial ELISA kits. TNF-α (BD Bioscience, Franklin Lakes, NJ, USA), IFN-gamma (BD Bioscience, Franklin Lakes, NJ, USA), and IL-2 (Biosource) concentrations were assayed. Samples were run in triplicate, and results are expressed as cytokine concentration (pg mL^{-1}).

Protein extraction and western blot analysis

The samples were homogenized in ice-cold buffer consisting of 0.1 % sodium dodecylsulfate, 0.1 % sodium deoxycholate, 1 % Triton X-100 in phosphate-buffered saline with freshly added protease inhibitors (phenylmethylsulfonylfluoride, aprotinin, leupeptin, 1,10-phenanthroline, iodoacetamide, and ortho-vanadate). The protein content was measured by the bicinchoninic acid assay, using BSA as standard. Samples were boiled for 5 min in Laemmli buffer, separated by 10 % SDS–PAGE, electroblotted onto nitrocelulose membranes, and blocked with 5 % (w v^{-1}) nonfat dry milk in Tris-buffered saline. After incubation with the proper antibodies, the bands were detected by enhanced chemiluminescence (PerkinElmer, Madrid, Spain). Bands quantitated using the National Institutes of Health software Scion Image.

Analysis of gene expression by reverse transcriptase-PCR analysis

Total RNA was obtained by the Trizol method (Invitrogen, Barcelona, Spain). One μg of RNA was retrotranscribed, and specific RNA sequences were amplified with a Stratagene MX3005P real time PCR device using the following primers: AAT GAC CTG TTC TTT GAG GCT G/CGA GAT GCT GCT GTG AGA TTT (IL-1β), TAC TGA ACT TCG GGG TGA TTG/CAG CCT TGT CCC TTG AAG AGA (TNF), and CCA TTG GAG GGC AAG TCT GGT G/CGC CGG TCC AAG AAT TTC ACC (18S rRNA subunit).

Statistical analysis

Results were expressed as mean ± SEM. Differences among means were tested for statistical significance by one-way ANOVA and a posteriori Fisher least significance difference test. Analyses were carried out with the SigmaStat 3.5 program (Jandel Corporation, San Rafael, CA, USA). Differences were considered significant at $P < 0.05$.

Results

Body weight evolution is shown in Fig. 1 (relative weight gain is referred to day 0, that is, when colitis was induced, for better clarity). Animals exhibited the normal trend to gain weight up until day-1 (not shown), when they were fasted prior to colitis induction. From that point on, the control group resumed its previous pace, while all colitic groups recovered only slowly, as expected. Of note, the average gain in both AHCC + probiotic groups was higher immediately after colitis induction compared with the TNBS group ($P < 0.05$ for days 2 and 3 in both

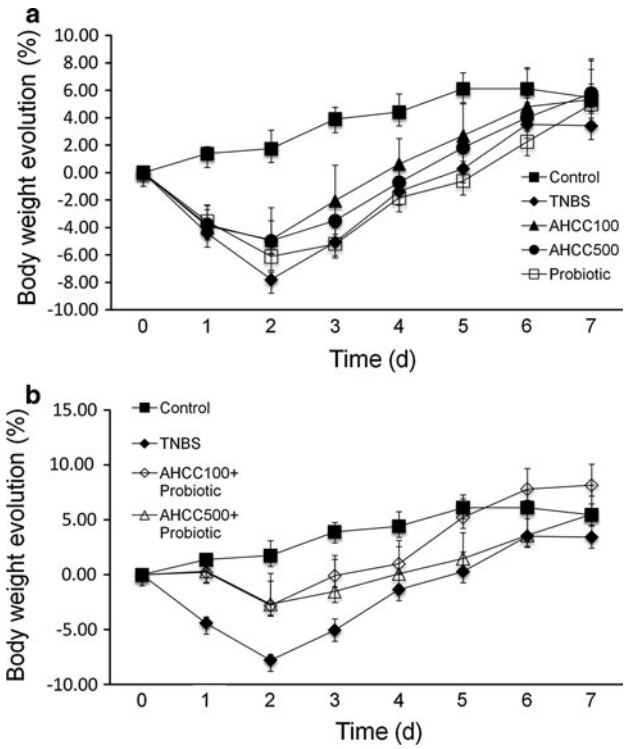

Fig. 1 Percent body weight gain after TNBS colitis induction. For better clarity the results are presented in two separate *graphs*, showing the individual (**a**) or combined treatments (**b**). All groups differ from the control group (not shown). $^{*}P < 0.05$ versus TNBS group

AHCC + probiotic groups, and additionally, in day 4 for the AHCC100 + probiotic). The AHCC 100 + probiotic group appeared to have the fastest recovery, even overshooting the Control by the end of the experiment ($P > 0.05$). Consistent with previous studies, by the end of the week, colitic animals had reached body weight values similar to those of the controls. Food intake generally paralleled these changes, although it tended to be somewhat higher in the AHCC100 + probiotic group ($P = 0.120$ for the first 2 days post-induction) (Table 1). No differences were noted on water consumption among the groups (data not shown).

In line with previous experiments, the large bowel was thickened by the inflammatory response and fibrosis, resulting in an approximately 2.5-fold increase in the colonic weight–length ratio (Table 1). This was substantially lower in the AHCC500 (50 %) and the AHCC100 + probiotic groups (71 %), with the latter reaching significance. These changes were due chiefly to changes in colonic weight, with a minor contribution of reduced length shortening (not shown). The macroscopic damage score was correspondingly augmented in all animals receiving TNBS (Table 1). The score was lower in the AHCC500 and the AHCC100 + probiotic groups (37 and 24 %, respectively) but without reaching significance. TNBS control rats exhibited approximately 15 % of total colonic surface affected by epithelial necrosis, as assessed by image analysis (Table 1), and again, this was lower in these 2 groups (40 and 60 %, respectively, $P = 0.1$–0.2).

Colitis status was next assessed biochemically, by the measurement of tissue MPO and AP enzymatic activities. MPO is a widely used index of leukocyte (neutrophil) infiltration [2], and it was dramatically augmented by TNBS colitis (Fig. 2). This increase was approximately halved in the AHCC groups ($P > 0.05$) and further reduced in the probiotic and both AHCC + probiotic groups ($P < 0.05$). AP activity is another marker of colonic inflammation, characterized by our group and others [3–5], which is increased as a result of changes in enzyme expression at the epithelial levels and leukocyte infiltration. These changes are associated to an augmented sensitivity to inhibition by the specific blocker, levamisole. Our results (Fig. 3) show that, consistent with previous observations, AP colonic activity and sensitivity to levamisole are increased in the TNBS group in this experiment. This was unaffected in the AHCC groups, whereas rats treated with probiotic (±AHCC) exhibit intermediate values between colitic and control rats.

Table 1 Macroscopic damage parameters values of TNBS colitic rats and food intake

	Damage score (arbitrary units)	Necrosis area (%)	Colon weight/length ratio (mg cm^{-1})	Food intake (g rat^{-1})	
				Day 1	Day 2
Control	0 ± 0^{a}	0 ± 0^{a}	70.1 ± 4.5^{a}	29.2	26.9
TNBS	8.8 ± 1.7^{b}	15.1 ± 4.5^{b}	195.0 ± 48.6^{b}	13.9	5.6
AHCC100	9.7 ± 1.8^{b}	13.8 ± 4.3^{b}	187.0 ± 31.7^{b}	12.8	11.8
AHCC500	5.5 ± 2.2^{b}	9.1 ± 4.6^{b}	133.0 ± 31.2^{b}	12.4	11.5
Probiotic	9.9 ± 1.3^{b}	12.5 ± 3.1^{b}	166.0 ± 25.6^{b}	11.7	7.7
AHCC100 + probiotic	6.7 ± 1.9^{b}	6.1 ± 2.7^{b}	106.0 ± 14.5^{c}	16.9	12.8
AHCC500 + probiotic	7.2 ± 2.6^{b}	14.0 ± 6.0^{b}	158.0 ± 43.5^{b}	12.2	9.4

Values are means ± SE except food intake. Means without a common letter differ ($P < 0.05$)

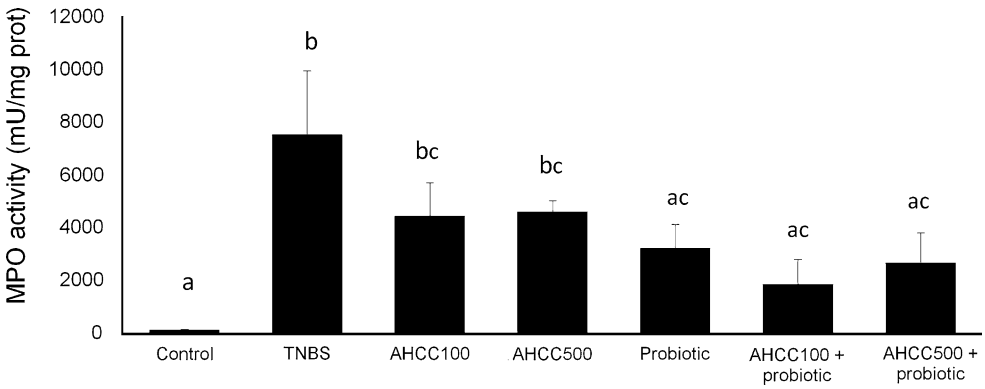

Fig. 2 Colonic myeloperoxidase activity in rat TNBS colitis. Rats were treated with AHCC at two different doses with or without *B. longum* BB536 for 7 days (or the corresponding controls) prior to colitis induction and daily thereafter and their MPO activity measured in longitudinal colon samples. Means without a *common letter* differ ($P < 0.05$)

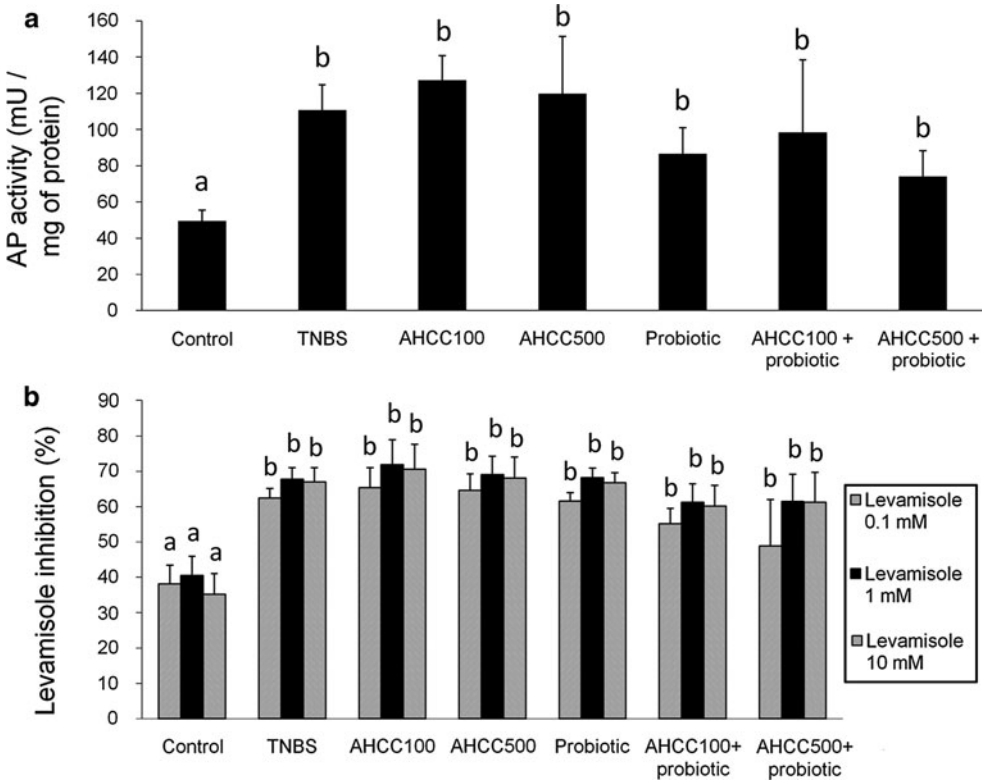

Fig. 3 Colonic alkaline phosphatase activity (**a**) and sensitivity to levamisole (**b**) in rat TNBS colitis. Rats were treated with AHCC at two different doses with or without *B. longum* BB536 for 7 days (or the corresponding controls) prior to colitis induction and daily thereafter and their AP activity measured in longitudinal colon samples. Inhibition by levamisole was also assessed. Means without a *common letter* differ ($P < 0.05$)

Next, we focused on iNOS and COX-2 expression, as measured by Western blot in colonic tissue. TNBS colitis resulted as expected in a significant upregulation of these proteins (Figs. 4, 5). Interestingly, the only groups showing any differences at this level were the AHCC100 (±probiotic) groups. As shown in these figures, the fact that the upshot in COX-2 was higher may have facilitated the significant differences observed with the AHCC100 groups.

IL-1β mRNA levels in the colonic tissue were measured by RT-PCR, using 18S as reference gene. As shown in Fig. 6, there was a marked increase in the TNBS colitic group, while all the treated groups exhibited values comparable with the control group. It should be noted that these differences did not reach statistical significance due to the relatively high variability in the TNBS group.

Finally, we examined IL-2, TNF-α and IFN-γ production by mesenteric node cells (mostly lymphocytes) in primary culture ex vivo, both under basal and concanavalin A-stimulating conditions (Fig. 7). In all cases, a heightened state of secretion was observed in the TNBS group in both basal and stimulatory conditions, although the effect was generally moderate. Basal IL-2 and IFN-γ secretion was lowered in all treatment groups, although with different significance levels (i.e. $P < 0.05$ only for IFN-γ). There was no effect of any treatment on TNF-α secretion. Under concanavalin A stimulation, all colitic groups tended to show similar values, although in some instances cytokine release was actually higher in the treated groups.

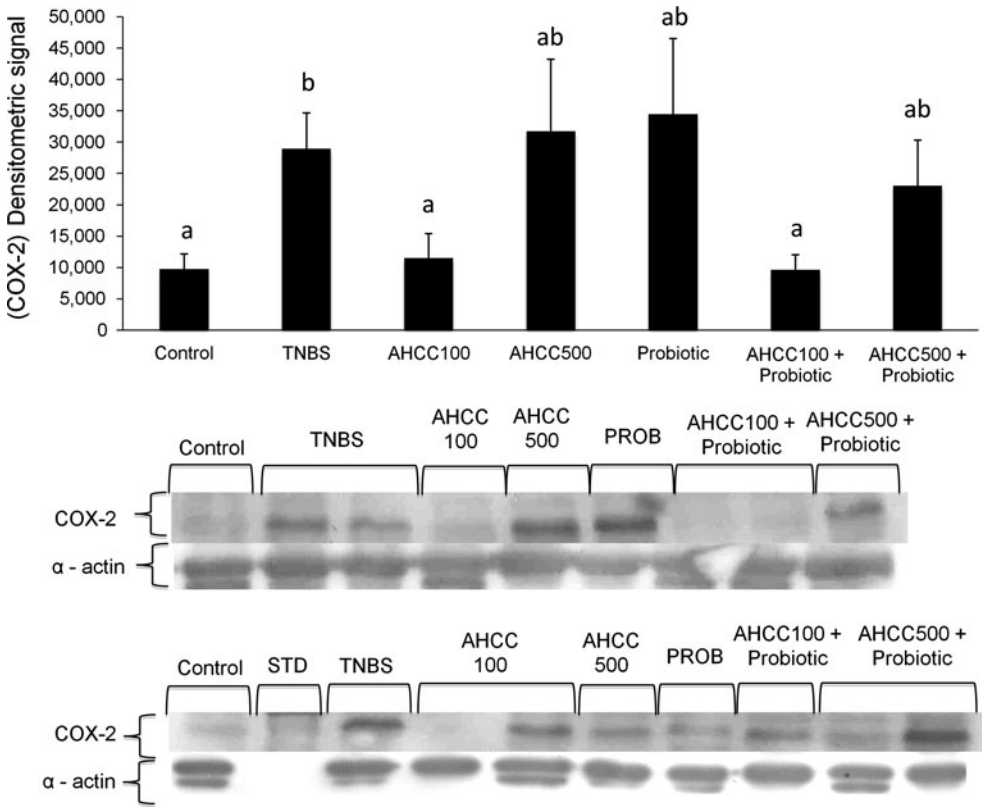

Fig. 4 Colonic expression of COX-2 rat TNBS colitis. Rats were treated with AHCC at two different doses with or without *B. longum* BB536 (or the corresponding controls) for 7 days prior to colitis induction and daily thereafter. Colonic expression of COX-2 was measured by western blot in longitudinal colon samples and quantitated with Scion Image. Means without a *common letter* differ ($P < 0.05$)

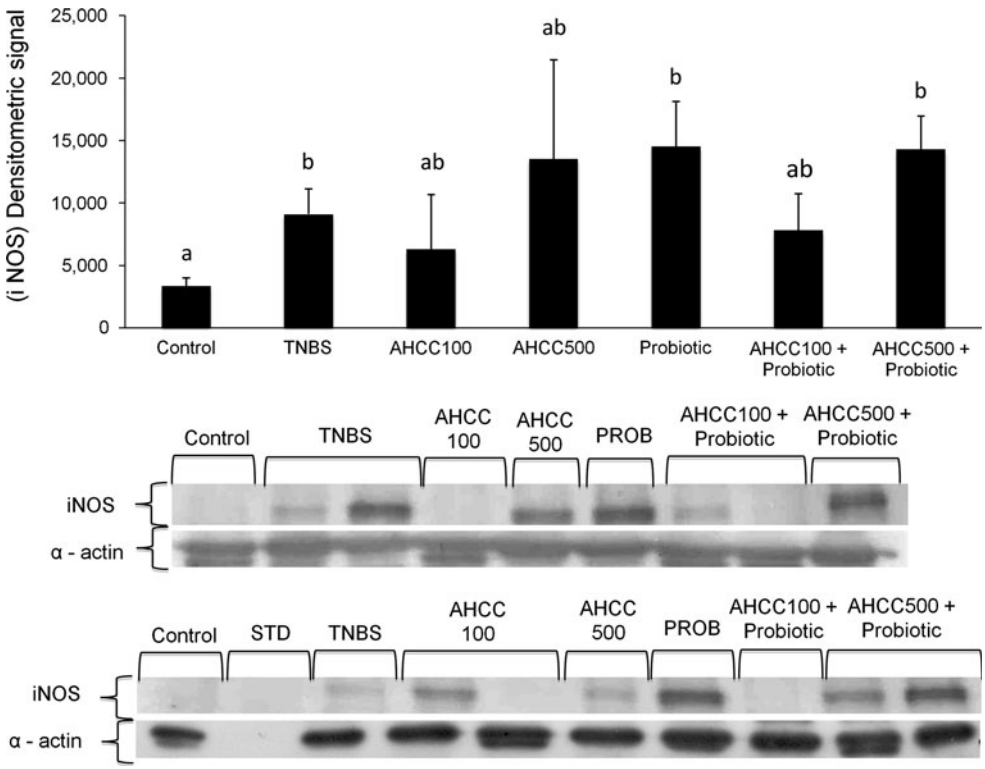

Fig. 5 Colonic expression of iNOS in rat TNBS colitis. Rats were treated with AHCC at two different doses with or without *B. longum* (or the corresponding controls) for 7 days prior to colitis induction and daily thereafter. Colonic iNOS expression was measured by western blot in longitudinal colon samples and quantitated with Scion Image. Means without a *common letter* differ ($P < 0.05$)

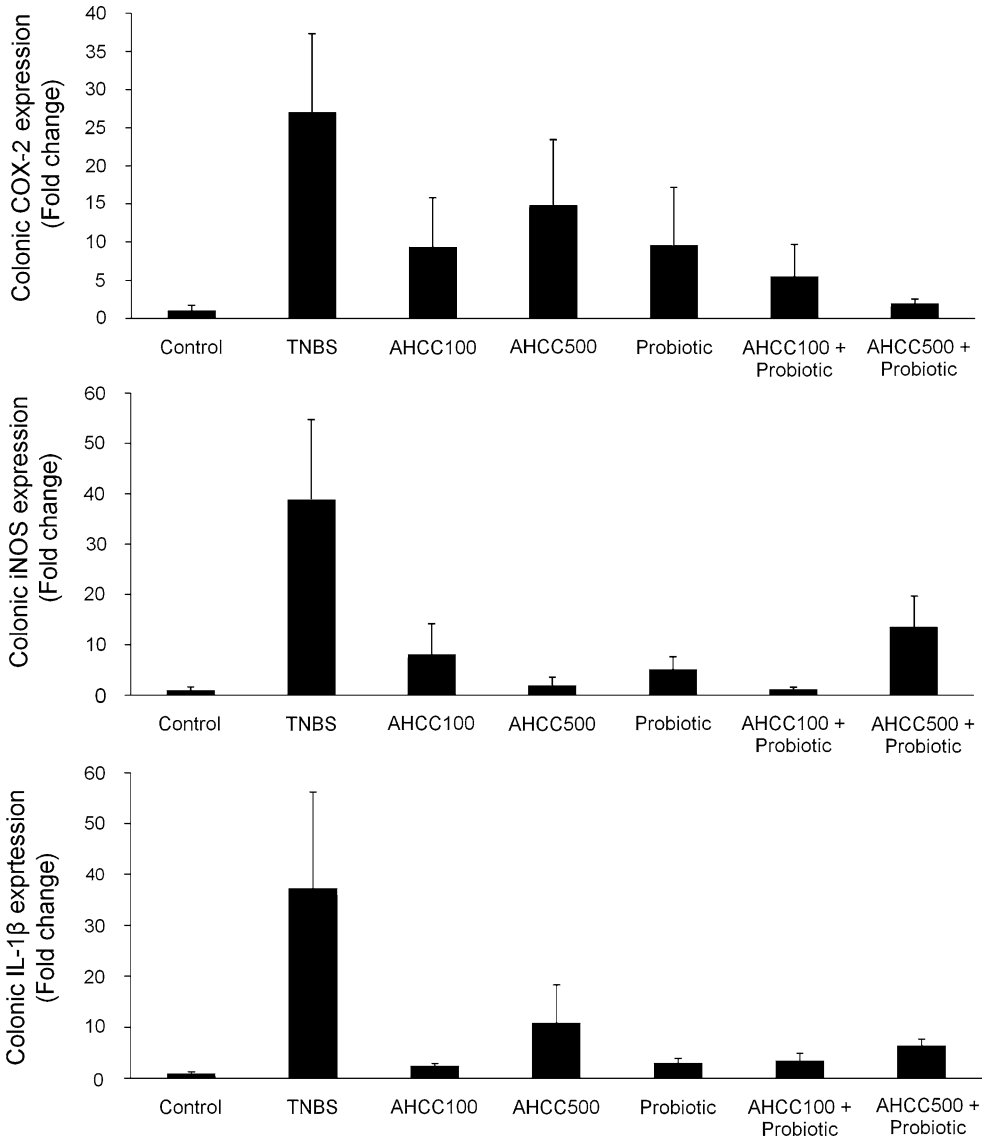

Fig. 6 Colonic mRNA expression of COX2, iNOS, IL-1β in rat TNBS colitis. Rats were treated with AHCC at two different doses with or without *B. longum* BB536 (or the corresponding controls) for 7 days prior to colitis induction and daily thereafter. Colonic COX2, iNOS and IL-1β expression was measured by RT-PCR in longitudinal colon samples. No significant differences were detected

Discussion

The present study was designed to test the hypothesis that AHCC and *B. longum* may act synergistically in intestinal inflammation. This type of experiment requires (1) that AHCC has anti-inflammatory effects in the colon, as already established by us; (2) that *B. longum* BB536 exerts beneficial effects by itself in the same conditions (or is at least neutral); and (3) that combined treatment results in enhancement of the therapeutic effect. The first requisite is substantiated not only by the previous published data but also by the results obtained in the present experiment, although the magnitude of the therapeutic effect was somewhat lower than expected in the present study. In general, AHCC treatment at the dose of 500 mg kg^{-1} produced a 40–50 % reduction in inflammatory parameters such as colonic damage score, weight to length ratio, necrotic area or MPO activity. Some of these changes were not significant, due to the fact that the design includes 7 groups, thus reducing statistical power. In this study, we assayed also a lower 100 mg kg^{-1} dose, which had not been tested before in an IBD model. As expected, this group lost much of the effect attributable to AHCC 500 mg kg^{-1}, but surprisingly it was only at the lower dose when COX-2 was substantially lowered. The reason for this particular effect is unknown.

The second requisite is not as immediate, since we had no prior experience with this bacterial species. However, this is a well-known probiotic. Thus, *B. longum* HY8004 inhibits glycosaminoglycan degradation by intestinal bacteria and ameliorates TNBS colitis in mice [10]. Some evidence in this regard pertains to mixes of probiotic strains featuring *B. longum* [11]. *B. longum* is part of the well-known probiotic mix VSL#3, composed also by four

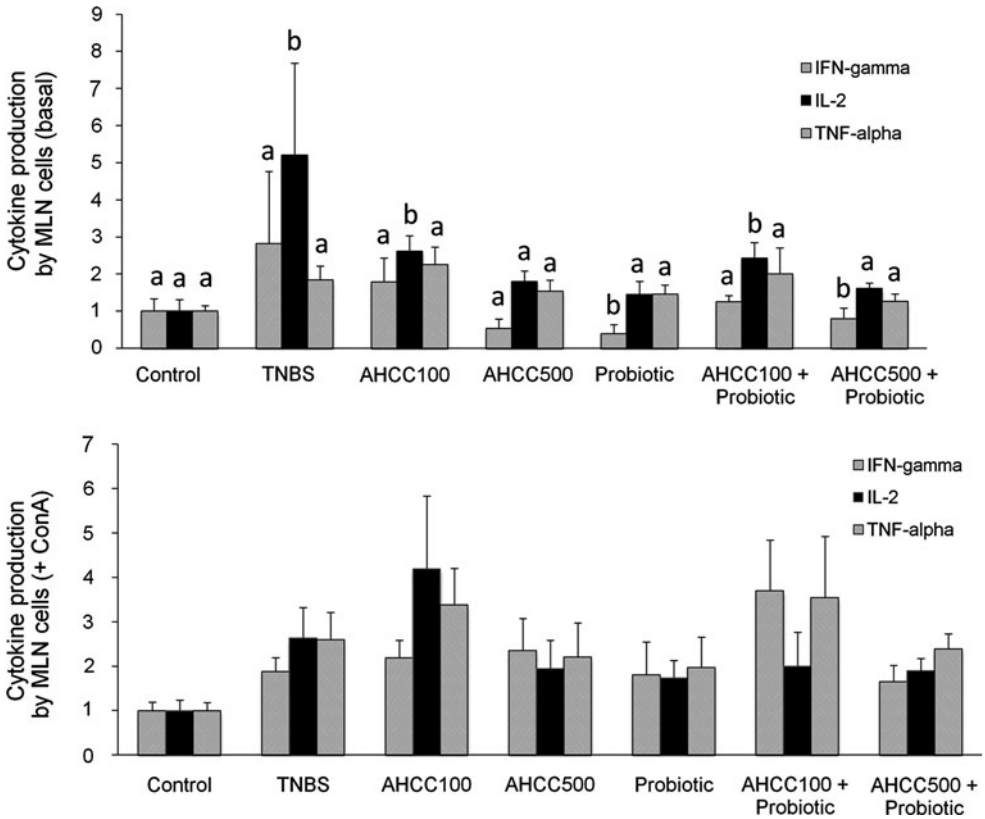

Fig. 7 Production of cytokines by mesenteric node cells cultured ex vivo in rat TNBS colitis. Mesenteric node cells were obtained at the time of animal sacrifice and cultured for 48 h with or without ConA. Cytokine levels were measured by ELISA. Basal levels (±ConA) were: 52.5/1,1161.2 pg mL^{-1} (IFN-γ), 33.7/1,431.3 pg mL^{-1} (IL-2) and 5.2/146.2 pg mL^{-1} (TNF-α). Means without a *common letter* differ ($P < 0.05$)

strains of lactobacilli (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. bulgaricus), two strains of bifidobacteria (*B. breve* and *B. infantis*), and *Streptococcus salivarius* subsp. thermophilus. Probiotics have been advocated for a number of gastrointestinal conditions, including traveler's and antibiotic related diarrhea, irritable bowel syndrome and IBD. They may be useful also as adjuvants to preexisting therapy. For example, VSL#3 in combination with low-dose balsalazide (a prodrug of mesalazine [mesalamine; 5-aminosalicylic acid]) was more effective than standard doses of balsalazide or mesalazine monotherapy in the treatment of acute mild to moderate ulcerative colitis [12]. In the present study, *B. longum* BB536 was used at a dose of 5×10^6 CFU rat^{-1} day^{-1}, which was selected on the basis of previous pilot experiments, suggesting that this was on the lower end of the dose response curve. Indeed this intervention resulted in significant reduction in colonic MPO activity and basal IL-2 and IFN-γ secretion by mesenteric node cells but no amelioration of other parameters, with the possible exception of colonic AP activity.

Our study design included 2 symbiotic groups, with 2 different AHCC (prebiotic) doses and a fixed probiotic dose. This was intended in order to leave some room for the potential synergistic effect to show. Indeed, there appears to be a limit to the therapeutic benefit achievable by pharmacological or nutritional interventions in this (and other) IBD models, so that the use of 'optimal' doses of individual treatments may obscure the observation of true synergistic interaction. Our data indicate that the benefit obtained with the combination of AHCC at 100 mg kg^{-1} and *B. longum* BB536 is superior to that of individual treatments in terms of body weight gain, colonic weight to length ratio, MPO activity and iNOS expression, and appears close in terms of colonic damage score and necrosis. However, colonic MPO activity was not substantially lowered further than with the probiotic alone, an example of the 'limit' noted above. Similarly, iNOS expression was comparable in the AHCC100 + probiotic and AHCC100 groups. It is interesting to note how in these two cases the combined treatment overcomes the limitations of the prebiotic and probiotic. When the higher dose of AHCC was associated with *B. longum* BB536, there was a better control of colonic MPO and AP activities (the latter nonsignificant) than with the prebiotic alone but not of colonic weight to length ratio or necrosis. Colonic damage score and mesenteric node cell IL-2 and IFN-γ secretion were similar in the AHCC500 and AHCC500 + probiotic groups. Therefore, the association is still beneficial with the high dose of AHCC, but it appears to be inferior to that with AHCC100. It should be noted than some of this loss of effect may be an epiphenomenon, for instance with colonic weight to length ratio, and that some gain of effect fails to reach significance due to the complex design (see AP

activity). However, it is possible that synergism is dependent on the use of certain doses (see below).

A classical concept in pharmacology is that synergism, that is, a potentiation of therapeutic effects when 2 drugs are given in combination rather than separately, arises when the mechanisms of the respective treatments are different and complementary. The use of prebiotics and probiotics was based originally on the importance of the colonic flora for gastrointestinal health and specifically for IBD. In general, probiotics are considered host-friendly bacteria that somehow may dampen inflammation, possibly by counteracting the effects and/or growth of potentially pathogenic species, although how this actually occurs is unclear. The composition of the flora is so important that flora transplant has been used successfully to treat *Clostridium difficile* diarrhea. In vitro, *B. longum* BB536 appears to inhibit immune responses, including lowering cytokine release and reducing lamina propria mononuclear cell number [13]. More research is needed to clarify the role of probiotics for preventing antibiotic-associated diarrhea, *C. difficile* infection, travelers' diarrhea, irritable bowel syndrome, ulcerative colitis, Crohn's disease, and vulvovaginal candidiasis [3].

On the other hand, prebiotics are undigestible oligosaccharides that reach the colon essentially intact, where they somehow favor the selective growth of host-friendly bacteria (including any probiotics present of course). In addition, prebiotics may act by lowering intestinal pH, reducing mucosal attachment of bacteria and modifying the host immune response. They may be useful in other conditions such as upper respiratory infections [14]. On the basis of the complementarity of mechanism, it is expected that prebiotic and probiotics act synergistically, and therefore this type of combination has a specific name: synbiotic. Synbiotic *B. longum* associations have been claimed to offer benefits for quality of life in IBD patients [15]. *B. longum* has been tested short term in UC patients in association with a prebiotic substrate called Synergy 1, with positive results [16].

Our results support the synbiotic association AHCC + *B. longum* BB536 for IBD, although some points should be remembered. First, the combination has been established to be beneficial at the doses used, namely 5×10^6 CFU of *B. longum* BB536 and 100 (or 500) mg kg^{-1} of AHCC. The fact that some effect may be lost at the higher dose of AHCC and the absence of data with other doses suggest that the therapeutic effects are probably dose dependent, and this has to be considered for clinical use. Both AHCC ?tul?> and *B. longum* (including the BB536 strain) are used widely in humans as functional foods, and they are considered nontoxic. On a body surface basis, the equivalent doses would be 2×10^8 CFU and 1.1 g AHCC per day, which are within the intake range currently used for both. Second, although the combination has been tested in a well-known IBD model that is used profusely to test different therapies at the preclinical level, care must be taken at extrapolating the results to the clinical arena.

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