

# Active Hexose Correlated Compound Acts as a Prebiotic and Is Antiinflammatory in Rats with Hapten-Induced Colitis<sup>1,2</sup>

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## Abstract

Active hexose correlated compound (AHCC) is a product prepared from the mycelium of edible *Basidiomycete* fungi that contains oligosaccharides. Here we have studied the antiinflammatory effect of AHCC in the trinitrobenzenesulfonic acid (TNBS) model of colitis in rats. Rats received AHCC (100 or 500 mg/kg) daily starting 2 d before (pretreatment) colitis induction and were killed 6 d after the TNBS challenge. The status of the rats was assessed by morphological and biochemical methods. The effect of AHCC on the colonic microflora was also assessed by studying the bacteria profile in feces by standard culture techniques. AHCC administration attenuated colonic inflammation, improving rat weight, food intake, damage score, extension of necrosis, colonic weight, colonic weight-to-length ratio, myeloperoxidase and alkaline phosphatase activities, glutathione concentration, and the expression of proinflammatory cytokines and chemokines (IL-1 $\beta$ , IL-1 receptor antagonist, TNF, and monocyte chemoattractant protein-1) and of mucins 2–4 and trefoil factor 3. The magnitude of the antiinflammatory effect of AHCC was similar to that of sulfasalazine (200 mg/kg). The study of colonic microflora indicated that rats treated with AHCC had higher aerobic and lactic acid bacteria counts as well as higher bifidobacteria counts, whereas clostridia were reduced when compared with the TNBS group. Therefore, our results indicate that AHCC is antiinflammatory and could be useful as a prebiotic to design functional foods for inflammatory bowel disease patients. *J. Nutr.* 137: 1222–1228, 2007.

## Introduction

Active hexose correlated compound (AHCC)<sup>5</sup> is a product prepared from the mycelium of edible *Basidiomycete* fungi that contains oligosaccharides (~74% of AHCC, ~20% being of the  $\alpha$ -1,4-glucan type), amino acids, lipids, and minerals (1). The acetylated forms of the  $\alpha$ -1,4-glucan type of oligosaccharides have low molecular mass (5 kDa) and are considered the main active ingredient of AHCC (2–4).

AHCC has been well tolerated both as a human nutritional supplement and as a therapeutic agent (1,5). Furthermore, several studies have proven that the oral administration of AHCC has a wide variety of therapeutic effects, including anticancer effects both in animal models and in clinical trials (2,3,6–8), prevention of the onset of diabetes induced by streptozotocin in animal models (9), prevention of liver injury produced by CCl<sub>4</sub> in mice (10), and improvement of the immune response (7,11,12). The anticancer activity of AHCC has been ascribed to its immunomodulatory and antioxidant properties (4,7,13).

Inflammatory bowel disease (IBD), which comprises ulcerative colitis and Crohn's disease, is characterized by the chronic and relapsing inflammation of the intestine, resulting in diarrhea, abdominal pain, and a variety of other symptoms. There are important differences with regard to pathophysiology and treatment between both IBD types. Namely, ulcerative colitis affects exclusively the large intestine at the mucosal level, whereas Crohn's disease is characterized by transmural inflammation and may involve any segment of the gastrointestinal tract. The importance of IBD as a health problem lies in its impact on the patient's quality of life and its substantial prevalence, which has increased in the last few years.

Although the etiology of IBD is currently unknown, it has become clear that the intestinal flora is determinant in the

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<sup>5</sup> Abbreviations used: AHCC, active hexose correlated compound; AHCC100, experimental group that received 100 mg·kg<sup>-1</sup>·d<sup>-1</sup> AHCC; AHCC500, experimental group that received 500 mg·kg<sup>-1</sup>·d<sup>-1</sup> AHCC; AP, alkaline phosphatase; C, control group; IBD, inflammatory bowel disease; IL-1ra, IL-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; MUC, mucin; SZ, experimental group that received sulfasalazine (100 mg·kg<sup>-1</sup>·d<sup>-1</sup>); T, TNBS group; TFF-3, trefoil factor-3; TNBS, trinitrobenzenesulfonic acid.

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development of the events that ultimately give rise to the relapses (14). Thus, IBD probably represents an uncontrolled and exacerbated response to luminal antigens that are innocuous for the normal population (15). Although IBD can often be successfully managed pharmacologically, the drugs used, such as corticoids, aminosalicylates, or azathioprine, have a plethora of serious adverse effects that limit their application. Hence, the search for new treatments with a low profile of adverse effects is much warranted (16). One such strategy involves the use of prebiotics and/or probiotics, with the objective of modulating the intestinal flora to promote the growth of host-friendly bacteria and inhibit the proliferation of potentially harmful microorganisms (15), although other mechanisms may be relevant in this regard (17).

Several studies have shown that oligosaccharides obtained from different sources (18,19) have an antiinflammatory effect in models of intestinal inflammation. Here, we present evidence of the colonic antiinflammatory effect of AHCC in a rat model of experimental intestinal inflammation induced by the hapten trinitrobenzenesulfonic acid (TNBS).

## Materials and Methods

Except where indicated, all reagents and primers were obtained from Sigma. We purchased Taq polymerase from Amersham Biosciences. AHCC was provided by Amino Up Chemical.

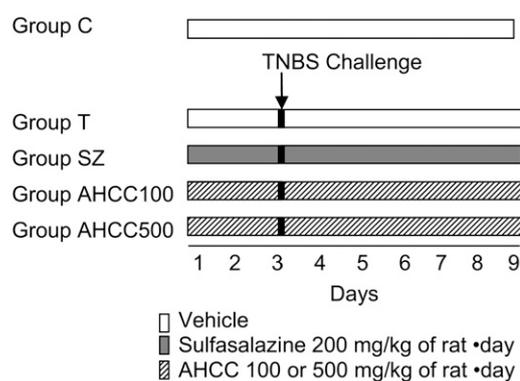
**Rats.** Female Wistar rats (200–250 g) obtained from the Laboratory Animal Service of the University of Granada were used, housed in macrolon cages, and maintained in our laboratory in air-conditioned animal quarters with a 12-h light-dark cycle. Rats were given free access to tap water and food. Diet composition (Panlab A04) 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 Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC).

**Induction of colitis.** Colitis was induced by the method of Morris et al. (20) with minor modifications. Briefly, rats were fasted overnight and anaesthetized with halothane. Under these conditions, rats were given 10 mg of 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.

**Experimental design.** Rats were randomly assigned to 5 different groups ( $n = 6$ ). Four groups [(T), SZ, AHCC100, and AHCC500] (Fig. 1) received the TNBS challenge to induce colitis as described above, and 1 group [control (C)] was given 0.25 mL of PBS intrarectally. Groups SZ, AHCC100, and AHCC500 received sulfasalazine (200 mg·kg<sup>-1</sup>·d<sup>-1</sup>) or 100 or 500 mg·kg<sup>-1</sup>·d<sup>-1</sup> AHCC in 1% methylcellulose orally, starting 2 d before the TNBS challenge, while groups T and C received the vehicle. Rats were treated until 6 d after the TNBS challenge, when the inflammatory response most closely resembles the human condition (21). An esophageal catheter was used to deliver all treatments. We determined food intake, water intake, and body weight 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–



**FIGURE 1** Experimental design. Groups T, SZ, AHCC100, and AHCC500 received the TNBS challenge to induce colitis and group C was given 0.25 mL of PBS intrarectally. Groups SZ, AHCC100, and AHCC500 received sulfasalazine (200 mg·kg<sup>-1</sup>·d<sup>-1</sup>), 100 or 500 mg·kg<sup>-1</sup>·d<sup>-1</sup> AHCC in 1% (w:v) methylcellulose p.o., starting 2 d before the TNBS challenge, whereas groups T and C received the vehicle. Rats were treated until 6 d after the TNBS challenge.

3), necrosis (0–5), and scarring and deformation (0–7) (22). The colon was subsequently divided longitudinally in several pieces for biochemical determinations. One of these pieces was used to measure TNF production, as indicated below. The sample for total glutathione content determination was immediately weighed and frozen in 1 mL of 5% (w:v) trichloroacetic acid (see below). The other fragments were immediately frozen in liquid nitrogen and kept at –80°C until used.

To determine the production of TNF, the fragments were weighed and incubated with cell culture medium (DMEM supplemented with inactivated bovine fetal serum [10% (v:v)], glutamine (2 mmol/L), penicillin (100 kU/L), streptomycin (0.1 g/L), and amphotericin B (2.5 mg/L) at 37°C for 24 h. After centrifugation (7000 × g; 10 min), the supernatants were used to determine the concentration of TNF by ELISA (Biosource). Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. (23), using 0.5% hexadecyltrimethylammonium bromide in Tris (pH = 6.0) for tissue homogenization and *o*-dianisidine dihydrochloride (533 μmol/L) as chromogen. The results are expressed as MPO milliunits per milligram wet tissue. Total glutathione content was quantitated with a recycling assay using 5,5′-dithiobis-2-nitrobenzoic acid as chromogen and glutathione reductase to regenerate oxidized glutathione (24). Results were expressed as nanomoles per milligram wet tissue. Alkaline phosphatase (AP) activity was measured spectrophotometrically, using disodium nitrophenylphosphate (5.5 mmol/L) as substrate in 50 mol/L glycine buffer with 0.5 mmol/L MgCl<sub>2</sub> (pH = 10.5) (25). Results are expressed as units per milligram protein.

**RT-PCR.** RT-PCR was used to examine the expression of mucins (MUC) 2, 3, and 4, trefoil factor 3 (TFF3), IL-1β, IL-1 receptor antagonist (IL-1ra), TNF, and monocyte chemoattractant protein-1 (MCP-1). Total RNA was extracted with Trizol (Life Technologies). A total of 5 μg of RNA per sample was subjected to reverse transcription using the First-Strand cDNA Synthesis kit (Amersham Biosciences). PCR amplification was performed using 1 μL of cDNA for a final PCR volume of 25 μL. The expression of the ribosomal 18 S unit was routinely examined as a loading standard. Rat primers were as follows: MUC2 (sense 5′-GCT CAA TCT CAG AAG GCG ACA G-3′; antisense 5′-CCA GAT AAC AAT GAT GCC AGA GC-3′); MUC3 (sense 5′-CAC AAA GGC AAG AGT CCA GA-3′; antisense 5′-ACT GTC CTT GGT GCT GAA TG-3′); MUC4 (sense 5′-CGT ACT AGA GAA CTT GGA CAT G-3′; antisense 5′-GGT AGG AGA ACT TGT TCA TGG-3′); TFF3 (sense 5′-ATG GAG ACC AGA GCC TTC TG-3′; antisense 5′-ACA GCC TTT TGC TGA CTG TA-3′); IL-1β (sense 5′-AAT GAC CTG TTC TTT GAG GCT G-3′; antisense 5′-CGA GAT GCT GCT GTG AGA TTT GAA G-3′); IL-1ra (sense 5′-GAG TCA GCT GGC CAC CCT G-3′; antisense

5'-CAC TGC TTC CCG AAT GTC TGA C-3'); TNF (sense 5'-TAC TGA ACT TCG GGG TGA TTG-3'; antisense 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'); MCP-1 (sense 5'-CAC TAT GCA GGT CTC TGT CAC G-3'; antisense 5'-CTG GTC ACT TCT ACA GAA GTG C-3'); ribosomal 18 S unit (sense 5'-CCA TTG GAG GGC AAG TCT GGT G-3'; antisense 5'-CGC CGG TCC AAG AAT TTC ACC-3'). The cycle numbers and hybridization temperatures for each PCR were as follows: 23 cycles and 56°C (for MUC2, MUC3, and TFF3); 27 cycles and 57°C (for MUC4, IL-1 $\beta$ , IL-1ra, MCP-1, and TNF); 17 cycles and 60°C (for the ribosomal 18 S unit). To set up the PCR conditions, different amounts of colonic RNA from a pool of samples were amplified using different numbers of cycles (data not shown). After the PCR amplification, 5  $\mu$ L of each reaction was resolved in 2.5% (w:v) agarose gels. Bands were quantitated with NIH software (Scion Image).

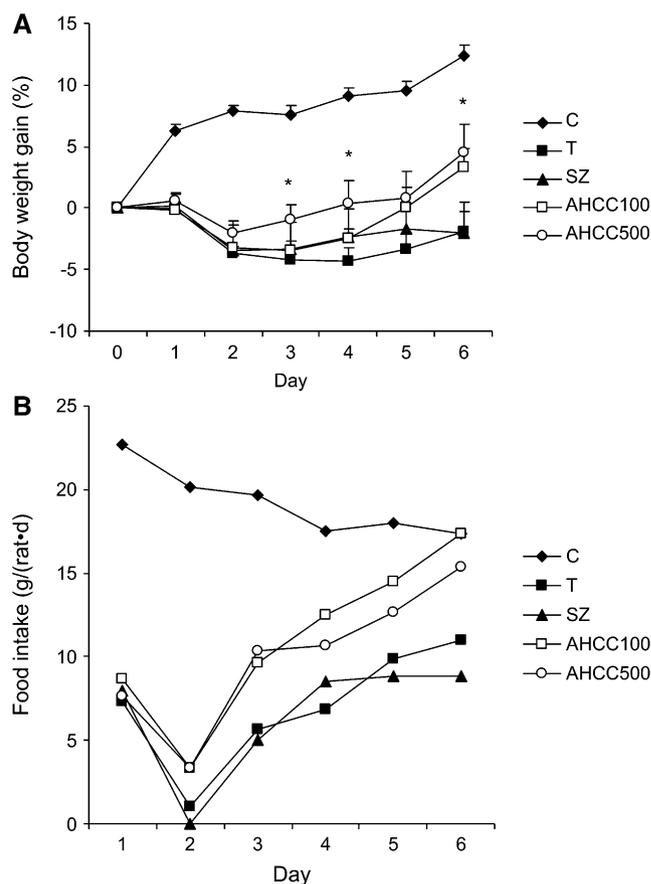
**Bacteriological analysis of feces.** A sample of feces was collected the day rats were killed and stored at -80°C until analysis. A total of 0.1 g of fecal material was weighed and homogenized with 0.9 mL PBS buffer containing 0.05% cysteine. A dilution series (10<sup>-1</sup>-10<sup>-6</sup>) was made and 10- $\mu$ L aliquots of the dilutions were plated on the different culture media: agar-blood (aerobes), MacConkey (enterobacteria), Wilkinson (anaerobes), Rogosa (lactic acid bacteria), Beerens (bifidobacteria), and reinforced clostridium agar medium (clostridia). The plates were subsequently incubated aerobically or anaerobically at 37°C for 3 d and all the colonies appearing on the appropriate dilution were counted. The results were expressed as the logarithm of the number of colony forming units.

**Statistical analysis.** The results were expressed as mean  $\pm$  SEM. Differences in means were tested for significance by 1-way ANOVA and a posteriori Fisher's least significance test except for bacterial counts that were analyzed by Kruskal-Wallis ANOVA. All analyses were the SigmaStat program (Jandel). Significance was set at  $P < 0.05$ .

## Results

As expected, colitic rats suffered anorexia and loss of body weight (Fig. 2). In addition, the administration of TNBS resulted in an increased damage score as a consequence of the observed mucosal erosion, epithelial necrosis, submucosal fibrosis, and edema. This was further reflected by an increase in colon weight and the colon weight-to-length ratio (Table 1). Colon pretreatment of rats with the higher dose of AHCC resulted in less pronounced anorexia and significantly increased relative body weight gain after the TNBS challenge (Fig. 2). At the macroscopical level, the administration of AHCC produced a decrease in the extent of necrosis, damage score, colon weight, and colon weight-to-length ratio (Table 1). Furthermore, the AHCC100 and AHCC500 groups were similar to the SZ group (Fig. 2; Table 1). This group of rats pretreated with SZ (200 mg $\cdot$ kg<sup>-1</sup> $\cdot$ d<sup>-1</sup>), an antiinflammatory drug, was included in the study as a positive control to assess the real therapeutic effect of AHCC.

The alterations induced by inflammation in the T group were reflected in the biochemical parameters assayed, with increases in the proinflammatory cytokines and chemokines IL-1 $\beta$ , IL-1ra, MCP-1, and TNF, as well as in MPO (a marker of neutrophilic



**FIGURE 2** Relative body weight gain (A) and food intake (B) after the TNBS challenge in rats. (A) Weight gains are relative to the day of TNBS challenge. Data are means  $\pm$  SEM,  $n = 6$ . \*Different from T,  $P < 0.05$ . All groups differed from C,  $P < 0.05$  (not shown). (B) Means of 6 rats per group and cage are shown.

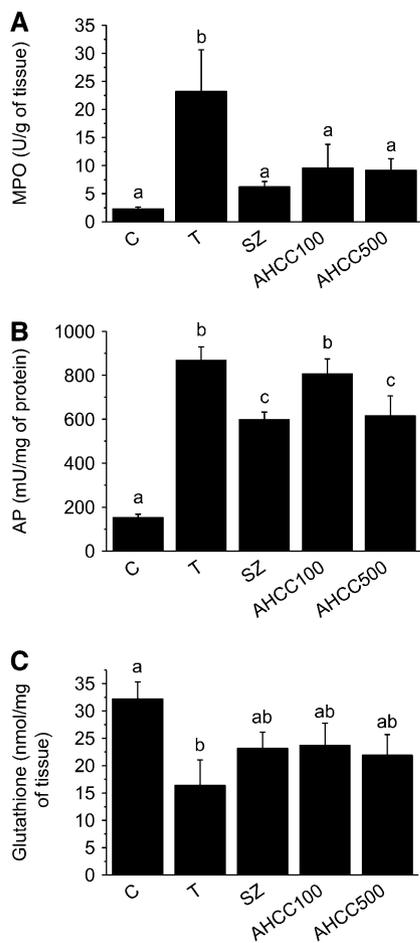
infiltration) and AP [a marker of inflammation (25)] activities (Figs. 3 and 4). Furthermore, the levels of glutathione, an endogenous antioxidant agent, were reduced in colitic rats (Fig. 4C). The administration of AHCC greatly affected the biochemical variables studied, indicating a reduction in the inflammatory status. Thus, as assayed by RT-PCR (Fig. 4), the administration of AHCC, independently of the dose studied, produced a dramatic reduction in the expression of IL-1 $\beta$ , IL-1ra, and TNF, which were normalized to control levels, as well as of MCP-1 (Fig. 4). These data are further supported by the decrease in the production of TNF by colon samples incubated in cell culture medium for 24 h (Fig. 5).

MPO activity also diminished with the administration of AHCC at both doses, whereas AP values were only slightly reduced and only at the highest dose assayed. Glutathione levels

**TABLE 1** Macroscopic indices of rat colonic inflammation in the different experimental groups<sup>1</sup>

	C	T	SZ	AHCC100	AHCC500
Colon weight, g	1.1 $\pm$ 0.1 <sup>c</sup>	2.8 $\pm$ 0.2 <sup>a</sup>	2.1 $\pm$ 0.4 <sup>b</sup>	1.9 $\pm$ 0.2 <sup>b</sup>	2.0 $\pm$ 0.3 <sup>b</sup>
Colon weight/length, mg/cm	56.8 $\pm$ 1.5 <sup>c</sup>	163.5 $\pm$ 9.4 <sup>a</sup>	127.3 $\pm$ 12.9 <sup>b</sup>	116.5 $\pm$ 10.7 <sup>b</sup>	119.6 $\pm$ 12.7 <sup>b</sup>
Extent of necrosis, cm	—	4.3 $\pm$ 0.4 <sup>a</sup>	2.6 $\pm$ 0.4 <sup>b</sup>	2.5 $\pm$ 0.4 <sup>b</sup>	2.6 $\pm$ 0.5 <sup>b</sup>
Damage score	—	10.8 $\pm$ 0.7 <sup>a</sup>	7.5 $\pm$ 1.5 <sup>b</sup>	6.2 $\pm$ 1.0 <sup>b</sup>	7.3 $\pm$ 1.2 <sup>b</sup>

<sup>1</sup> Values are means  $\pm$  SEM,  $n = 6$  for all groups. Means for a row without a common letter differ,  $P < 0.05$ .



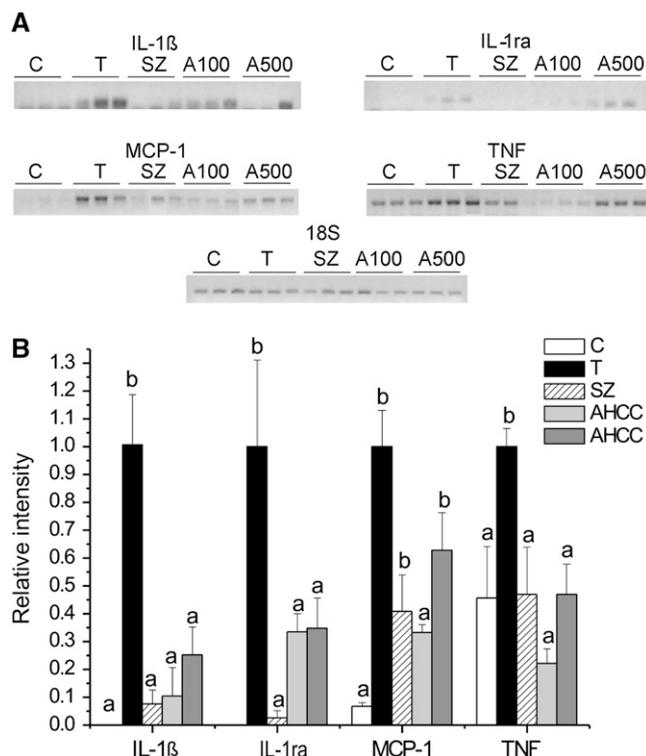
**FIGURE 3** Effect of AHCC pretreatment of rats with TNBS colitis on the colonic activities of MPO (A) and alkaline phosphatase (B) as well as on the colonic glutathione concentration (C). Data are means  $\pm$  SEM,  $n = 6$ . Means for a variable without a common letter differ,  $P < 0.05$ .

for all treated groups were between those of the C and T groups. In all cases, the effect of AHCC was comparable to that of sulfasalazine. To assess the effect of the treatment with AHCC on mucosal barrier defense, MUC and TFF3 expression was measured by RT-PCR. The induction of colitis produced an increment in the expression of MUC2–4 and TFF3 (Fig. 6). Pretreatment of rats with AHCC resulted in a normalization of these values, comparable again to that of sulfasalazine (Fig. 6).

To further investigate the mechanism of action of AHCC in the intestinal inflammation, a series of bacterial cultures were performed to determine its effect on fecal microbiota (Table 2). The administration of TNBS resulted in alterations in the microflora profile, decreasing both aerobic bacteria and lactic acid bacteria and increasing clostridia. AHCC normalized aerobic, clostridial, and lactic acid bacterial counts. In addition, AHCC increased the count of bifidobacteria. In contrast, sulfasalazine had no effect on aerobes or lactic acid bacteria, although its impact on bifidobacteria was more robust than that of AHCC.

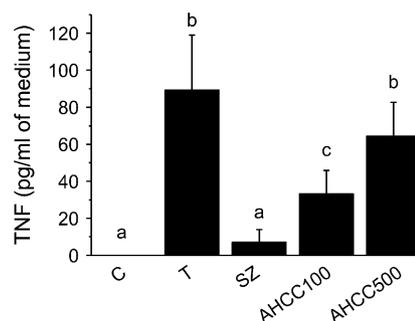
## Discussion

*Basidiomycete* mushrooms are usual components of the diet and AHCC is a product extracted from a myceloid of these

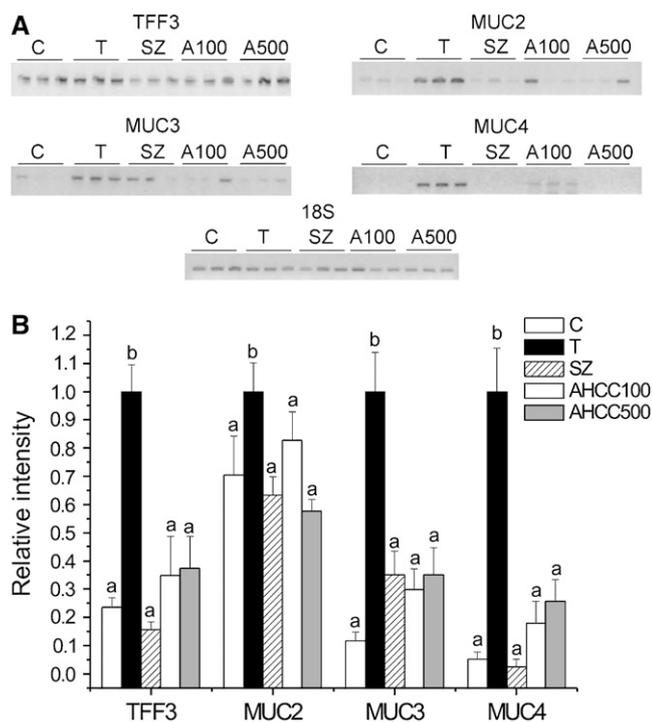


**FIGURE 4** Effect of AHCC pretreatment on the colonic expression of IL-1 $\beta$ , IL-1ra, MCP-1, and TNF in rats with TNBS colitis. Expression levels were determined by RT-PCR. Representative gels (A) and densitometric analysis using 18 S RNA as control (B) are shown. Data are means  $\pm$  SEM,  $n = 6$ . Means for a variable without a common letter differ,  $P < 0.05$ . A100, AHCC100; A500, AHCC500.

mushrooms. AHCC is composed mainly of oligosaccharides (~74%) and is enriched in low molecular weight acetylated  $\alpha$ -glucans. The biological effects of AHCC observed so far have been attributed to this glycan fraction (2,3,7). Because oligosaccharides from different sources are useful in the treatment of intestinal inflammation in several animal models (18,19), we hypothesized that AHCC could have therapeutic potential in IBD. Inflammation propitiates an increase in intestinal permeability, which may grant access to bacteria and/or bacterial products to the intestinal mucosal milieu, thus inducing or perpetuating an inflammatory immune response. Oligosaccharides may reduce inflammation by promoting the growth of the



**FIGURE 5** Effect of AHCC pretreatment on the production of TNF in colon fragments from rats with TNBS colitis. Fragments were weighed and incubated with cell culture medium for 24 h, then TNF was measured in the culture medium. Data are means  $\pm$  SEM,  $n = 6$ . Means for a variable without a common letter differ,  $P < 0.05$ .



**FIGURE 6** Effect of AHCC pretreatment on the expression of TFF3 and MUC 2–4 in colon fragments from rats with TNBS colitis. Expression levels were determined by RT-PCR. Representative gels (A) and densitometric analysis using 18 S RNA as control (B) are shown. Data are means  $\pm$  SEM,  $n = 6$ . Means for a variable without a common letter differ,  $P < 0.05$ . A100, AHCC100; A500, AHCC500.

normal intestinal flora while inhibiting the development of pathogen microorganisms (15,26–29). On the other hand, oligosaccharides were shown to bind to bacteria, inhibiting their attachment to intestinal cells and subsequent bacterial translocation (30–32). Other possible mechanisms include increased production of butyrate and stimulation of TLR by bacterial CpG DNA (19).

Here, we demonstrated that the administration of AHCC has an antiinflammatory effect in the TNBS preclinical model of IBD. Furthermore, our results indicate that the effect of AHCC is similar or even greater than that of SZ, a widely used drug to treat IBD. One of the most striking aspects of AHCC treatment was its beneficial effect on weight gain and food intake, in contrast with sulfasalazine, which had no effect. This effect could be related to the higher levels of IL-1ra associated with AHCC treatment compared with those of the sulfasalazine

group, because IL-1 $\beta$  participates in the anorexigenic response in this animal model (33,34). However, demonstration of this hypothesis will require measurement of blood protein levels. Alternatively, AHCC may interfere with other signaling pathways.

The antiinflammatory effect of both AHCC and sulfasalazine was further characterized by a reduction of the affected area and the intestinal edema, a decrease in MPO activity, and a reduction of proinflammatory cytokines. Leukocyte infiltration is characteristic of active/acute inflammatory intestinal lesions in IBD. The natural evolution of TNBS colitis is a progressive substitution of neutrophils by lymphocytes and macrophages (21). We have observed a reduction of the MPO activity and a strong inhibition of the synthesis of MCP-1 with both SZ and AHCC. The former may be interpreted as a sign of lower neutrophil infiltration, either as a result of reduced tissue injury or accelerated resolution of the inflammatory response. On the other hand, MCP-1 is produced by endothelial cells, smooth muscle cells, and macrophages and is 1 of the main cytokines implicated in monocyte recruitment. Although the low MCP-1 transcript levels are consistent with reduced monocyte infiltration, we cannot ascertain this without direct histological measurement. It is interesting to point out that MCP-1 is increased in IBD patients and its expression levels are related to the severity of the inflammation (35).

Alkaline phosphatase was also altered by the administration of AHCC. Recent studies carried out in our laboratory demonstrated that AP activity is a sensitive marker of intestinal inflammation (25). AP activity increased in the inflamed intestine as a result of both leukocyte infiltration and augmented epithelial enzyme activity, which in turn is associated with a change in isoform brought about by cellular stress. The administration of AHCC (500 mg·kg<sup>-1</sup>·d<sup>-1</sup>) decreased AP activity similar to that of the SZ group, indicating once again the antiinflammatory effect of the product. The fact that AP was not reduced at the lower dose of AHCC is intriguing and may indicate additional mechanisms operative at the dose of 500 mg·kg<sup>-1</sup>·d<sup>-1</sup>. On the other hand, both AHCC and SZ groups had colon glutathione concentrations intermediate to those of the control and TNBS groups. Although the statistical analysis could not establish a significant effect when compared to the TNBS inflamed rats, it seems likely that all treatment groups tend to have an improvement of the antioxidant defense status.

Both IL-1 $\beta$  and TNF are proinflammatory cytokines that play important roles in both IBD and TNBS colitis. The bioactivity of IL-1 $\beta$  is partly regulated by the endogenous inhibitor IL-1ra, which blocks the IL-1 receptor and prevents agonist binding (36). Mononuclear cells are the main source of

**TABLE 2** Effects of AHCC treatment on bacteria fecal levels in the different experimental groups of rats with TNBS colitis<sup>1</sup>

	C	T	SZ	AHCC100	AHCC500
	<i>Log CFU<sup>2</sup></i>				
Aerobes	7.83 $\pm$ 0.21 <sup>a</sup>	6.36 $\pm$ 0.16 <sup>b</sup>	6.79 $\pm$ 0.20 <sup>b</sup>	8.03 $\pm$ 0.21 <sup>a</sup>	7.58 $\pm$ 0.31 <sup>a</sup>
Anaerobes	8.34 $\pm$ 0.18	8.76 $\pm$ 0.21	8.16 $\pm$ 0.13	9.17 $\pm$ 0.23	8.58 $\pm$ 0.26
Lactic acid bacteria	7.46 $\pm$ 0.28 <sup>a</sup>	5.90 $\pm$ 0.35 <sup>b</sup>	5.50 $\pm$ 0.27 <sup>b</sup>	7.58 $\pm$ 0.37 <sup>a</sup>	7.00 $\pm$ 0.40 <sup>a</sup>
Bifidobacteria	5.99 $\pm$ 0.37 <sup>b</sup>	5.39 $\pm$ 0.12 <sup>b</sup>	8.21 $\pm$ 0.22 <sup>a</sup>	6.73 $\pm$ 0.48 <sup>a</sup>	6.84 $\pm$ 0.45 <sup>a</sup>
Clostridium	3.06 $\pm$ 0.26 <sup>b</sup>	4.52 $\pm$ 0.19 <sup>a</sup>	2.90 $\pm$ 0.65 <sup>b</sup>	3.46 $\pm$ 0.14 <sup>b</sup>	2.95 $\pm$ 0.14 <sup>b</sup>

<sup>1</sup> Values are means  $\pm$  SEM,  $n = 6$  for all groups. Means in a row with superscripts without a common letter differ,  $P < 0.05$ .

<sup>2</sup> Colony forming units.

both IL-1 $\beta$  and IL-1ra. On the other hand, the importance of TNF in IBD is illustrated by the fact that anti-TNF antibodies are widely used to treat ulcerative colitis and Crohn's disease. (37). Interestingly, AHCC administration reduced the mRNA levels of IL-1 $\beta$ , IL-1ra, and TNF, an effect comparable to that of sulfasalazine. However, the mechanism of this effect is probably different in both instances (see below).

Because prebiotics reportedly modulate MUC expression in the intestine (18,38), we assessed the MUC expression profile, as well as TFF3, by RT-PCR. The latter is a peptide implicated in the maintenance and healing of the intestinal mucosa, and MUC are a family of highly glycosylated proteins that, together with inorganic salts and water, form the mucus that protects epithelial surfaces, including the gastrointestinal tract. Colitis induction produced an increase in both TFF3 and MUC expression, indicating an epithelial reaction to heal mucosal wounds (39,40). The treatment with AHCC (and sulfasalazine) normalized these values, consistent with the less severe mucosal damage observed in these groups.

Finally, we explored the effect of AHCC on intestinal microflora by performing a bacteriological analysis of feces. It is generally accepted that intestinal microflora plays an important role in the pathogenesis of the intestinal inflammation and of IBD in particular. In fact, the mucosal inflammation characteristic of IBD is the culmination of a cascade of events and processes that promote barrier disruption and increase the absorption of luminal antigens from food and microorganisms, and bacterial products that stimulate different types of cells in the lamina propria to produce and release different proinflammatory mediators. Therefore, mucosal inflammation can be considered a self-perpetuating process in which disruption of the epithelial layer and absorption of luminal antigens play a central role. On the other hand, as stated above, prebiotics have been reported to promote the growth of host-friendly bacteria, reducing the growth of potentially harmful microorganisms. Our results indicate that the administration of TNBS decreased fecal aerobes, particularly lactic acid bacteria, and increased the counts of clostridium compared with the control group. The mechanism whereby TNBS modulates colonic flora is unknown and deserves further investigation. AHCC was able to normalize the microflora profile, increasing the counts for aerobic bacteria, lactic acid bacteria, and bifidobacteria while decreasing those of clostridium compared with the TNBS group. In contrast, sulfasalazine treatment only affected bifidobacteria and clostridia. Although sulfasalazine has some flora-modulating activity, this is not considered an important mechanism of action for this drug (41). Hence, the main determinants of AHCC antiinflammatory activity are likely to be the actions on aerobes and lactic acid bacteria. However, alternative mechanisms for AHCC unrelated to their prebiotic characteristics cannot be excluded based on the present data.

We tested 2 different doses of AHCC (100 and 500 mg·kg<sup>-1</sup>·d<sup>-1</sup>), which are in the high range for pharmacological products but in the low to midrange for nutritional/prebiotic products. Both doses were largely comparable in terms of anti-inflammatory efficacy. Although it may be worth testing lower doses of AHCC, the absence of apparent toxic effects and the therapeutic equivalence to sulfasalazine in this preclinical model makes it unlikely that the overall therapeutic value will be improved.

In conclusion, AHCC is antiinflammatory when administered as a pretreatment in the TNBS model of rat colitis, a widely used preclinical model of IBD. Its mechanism of action could be related to its ability to promote the presence of beneficial colonic

microflora (aerobic and lactic acid bacteria and bifidobacteria) while reducing clostridia. Therefore, this compound may be a valuable addition to current therapeutic alternatives to treat IBD. Further studies are warranted to validate this approach and to explore other possible mechanisms of action.

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