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Effects of Potential Prebiotics from *Codium fragile* on Intestinal Diseases

Su Won Oh [1,2], Sung Keun Kim [3], Byung Jae Ahn [2], Sung Kun Yim [2] and Seung Hwan Yang [1,*]

[1] Department of Integrative Biotechnology, Chonnam National University, Yeosu 59626, Republic of Korea; swoh@jbf.kr
[2] Marine Biotechnology Research Center, Jeonnam Bio Foundation, 21-7, Nonggongdanji 4Gil, Wando-eup, Wando-gun 59108, Republic of Korea; bjahn@jbf.kr (B.J.A.); skyim01@jbf.kr (S.K.Y.)
[3] Bada and Haecho Fishery Corp., 1111-2, Cheonma-ro, Pungyang-myeon, Goheung-gun 59548, Republic of Korea; wnbagirl@naver.com
* Correspondence: ymichigan@jnu.ac.kr

Abstract: This study examined the effects of an extract of the green algae *Codium fragile* (hereafter referred to as CFE) on dextran sulfate sodium (DSS)-induced colitis. As the administration of CFE increased, the proliferation of *Akkermansia muciniphila*, which is a key player in metabolic and gastrointestinal disorders, also increased. After CFE administration for 10 weeks, acetic acid was identified as the major metabolite in mouse cecum and β-glucuronidase activity in mouse fecesdecreased. Further, CFE significantly alleviated the acute intestinal injury induced by DSS administration, including DAI score, colon length, and histological score. The experimental group also displayed indications of significantly lower neutrophil activity and inflammation. In conclusion, the protective effect of CFE against DSS colitis suggests its clinical use by IBD patients.

Keywords: *Codium fragile*; *Akkermansia muciniphila*; inflammatory bowel disease (IBD); prebiotics

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1. Introduction

Recent studies have shown marine algae to possess a wide range of potential health applications, with moderating effects on the immune system being of particular interest in this study [1–5]. Additionally, algal metabolites such as sulfated polysaccharides and polyphenols have demonstrated multistep antiviral capability and provided a new route to develop new therapeutic methods to treat COVID-19 and other viral diseases [6].

Codium fragile is a traditional Asian food ingredient [7] mainly consumed in South Korea, Japan, and China. The main components of green algae are sulfated structural polysaccharides such as ulvans and sulfated cellulose, galactans, pectin, and mannans. These sulfated polysaccharides are not completely fermented by intestinal microbiota [8–10]. Some of the unfermented *C. fragile* polysaccharides have also been observed to have various interactions with biological systems [11,12]. These properties provide opportunities for the potential application of *C. fragile* polysaccharides as prebiotics.

The gut microbiota are microorganisms that live in the host's gastrointestinal tract. Human health is greatly influenced by the composition and metabolism of these microorganisms [13]. The microbiota in the human gastrointestinal tract have been studied extensively owing to their role both in pathogenesis and gut health maintenance. An important function of large intestinal microbiota is to break down substrates such as resistant starch and dietary fiber, which are not completely hydrolyzed by host enzymes in the small intestines [14–17]. Thus, health improvement via regulation of the gut microbiota has become an interesting research field.

Akkermansia muciniphila is a strictly anaerobic bacterium recently isolated from human feces. It uses mucin as the sole source of carbon and nitrogen elements [18]. Ottman et al. reported that *A. muciniphila* can utilize the mucin-derived monosaccharides fucose,

galactose, and *N*-acetylglucosamin [19,20]. The abundance of *A. muciniphila* in the feces appears to correlate with general gut health. The presence of *A. muciniphila* in feces has been associated with a healthy gut, and its abundance has been inversely correlated with several disease states. The abundance of *A. muciniphila* has been shown to be decreased in patients with ulcerative colitis and Crohn's disease [21,22]. However, as *A. muciniphila* is a strict anaerobe with highly limited growth conditions, there are currently no *A. muciniphila*-containing products in the world. Therefore, consuming prebiotics that can selectively promote *A. muciniphila* in the intestines is necessary.

Inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) are chronic intestinal diseases of unknown etiology [23,24]. Natural compounds have already shown promise as relatively safe therapeutic agents for the treatment and maintenance of IBD symptoms [25–27]. Algal polysaccharides are good candidates for the alleviation of intestinal inflammatory diseases due to their potential prebiotic efficacy [28,29].

In this study, we hypothesized that CFE may contain polysaccharides that exhibit therapeutic effects against intestinal inflammatory diseases. Until now, there have been few studies on the protective effects of CFE in mice with DSS-induced colitis. Thus, the present study was conducted to evaluate the protective effect of CFE against dextran sulfate sodium (DSS) colitis along with the proliferation of specific beneficial bacteria associated with a healthy intestine.

2. Materials and Methods

In this section, the methods for studying the effects of CFE on potential prebiotics and intestinal diseases are presented. Section 2.2 presents methods for evaluating prebiotics for growing specific beneficial bacteria important for maintaining intestinal health. In Section 2.3, methods for evaluating improvement in intestinal diseases are presented.

2.1. Preparation of CFE

CFE was prepared according to a previously described method [30]. In brief, previously collected *C. fragile* were washed, dried, and ground into powder. Boiling water was used to extract CFE from the powder, and the extract was concentrated and ultimately freeze-dried.

2.2. Potential Prebiotics for Improving Intestinal Diseases

2.2.1. Mouse Model

Seven-week-old male BALB/c mice (15–20 g) were obtained from KOSA BIO Inc. (Seongnam, Republic of Korea) and housed in A pathogen-free room (light cycle, 12 h light/dark; temperature, 22 ± 2 °C; humidity, 50 ± 5%). All mice were fed an AIN-93 diet (Research Diets, Inc., New Brunswick, NJ, USA) (Table 1), and sterilized water was provided *ad libitum* for a week during an adaptation period. Twenty mice were divided into four groups of five: (1) CTRL, fed a normal diet; (2) LCFE group, fed 75 mg of CFE per kg of body weight; (3) MCFE group, fed 150 mg of CFE per kg of body weight; (4) HCFE group, fed 300 mg of CFE per kg of body weight. Each group of mice was administered a daily oral dose of CFE dissolved in sterilized water for 10 weeks. The Mice were sacrificed via cervical dislocation under ether anesthesia. All procedures for this experiment were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC233-041) and the Ethical Committee of Experimental Animals in the Efficacy Evaluation Center of Berry & Biofood Research Institute (BBRI-IACUC-21001).

Table 1. Detailed dietary composition of AIN-93.

Ingredient	g/kg
Casein	200.00
Cornstarch	397.486

Table 1. *Cont.*

Ingredient	g/kg
Dextrose	132.00
Sucrose	100.00
Cellulose	50.00
Soybean oil	0.014
t-Butylhydroquinone	0.014
Salt Mix	35.00
Vitamin mix	10.00
L-Cystine	3.00
Choline Bitartrate	2.50

2.2.2. Real-Time PCR Quantification

Fecal samples from individual mice were collected in sterilized tubes and stored at −80 °C. Genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The primers used to detect *A. muciniphila* and *S. aureus* were based on 16S rRNA gene sequences retrieved from the National Center for Biotechnology Information databases using the Entrez program. Forward primers and reverse primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) (Table 2).

Table 2. 16S rRNA gene-targeted bacteria-specific primers used in this study.

Target		Primer	Primer Sequence (5′–3′)
Probiotics	*Bifidobacterium* spp.	Forward	CTCCTGGAAACGGGTGG
		Reverse	GGTGTTCTTCCCGATATCTAC
	Akkermansia muciniphila	Forward	CAGCACGTGAAGGTGGGGAC
		Reverse	CCTTGCGGTTGGCTTCAGAT
Pathogens	*Staphylococcus aureus*	Forward	GCCCCTTAGTGCTGCAGCTA
		Reverse	AGTTTCAACCTTGCGGTCGTA
	Clostridium spp.	Forward	TTGAGCGATTTACTTCGGT
		Reverse	CCATCCTGTACTGGCTCAC

PCR amplification was carried out in a total volume of 25 µL containing 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), both primers (10 pmol each), 50 ng purified target DNA, and final BSA concentration of 0.1 mg/mL (New England Biolabs, Ipswich, MA, USA). A StepOne Plus RT-PCR system (Applied Biosystems) was used for amplification and detection. The amount of genomic DNA extracted was determined using an ultraviolet spectrophotometer at 260 nm. In the PCR assay, we compared different amounts of bacterial DNA extracted from fecal samples to overcome bias due to inhibitory compounds such as bile salts. Each assay was performed in duplicate in the same run. The cycle threshold (CT) was calculated as the cycle number at which the reaction became exponential. The CT of each sample was then compared with a standard curve made by diluting genomic DNA (10-fold dilution) from cultures of the target bacterium.

2.2.3. Short Chain Fatty Acid (SCFA) Analysis

Cecum contents were stored at −80 °C for measurement of SCFA concentration. Acetic acid and butyric acid concentrations were determined by flame ionization detection on an Agilent 7890A GC-MS equipped with a DB-FATWAX Ultra Inert column 30 m × 530 mm × 0.25 µm (Agilent Technologies, Santa Clara, CA, USA).

2.2.4. β-Glucuronidase Activity Analysis

β-glucuronidase activity was analyzed using the method described by Goldin et al. [31]. Fecal samples were incubated at 37 °C in the wells of a microtiter plate, and then 10 mL

of sample was added in duplicate. Next, 100 µL of substrate solution was added, and incubated at 37 °C for 60 min. Next, 500 µL of 0.5 N NaOH was introduced to stop the reaction. Protein activity was quantified by measuring the absorbance at 405 nm. Triplicate assays were performed for each effector, and the mean values and standard deviations were reported. The concentration of 4-nitrophenol was determined using a standard curve of 4-nitrophenol in sodium phosphate buffer.

2.3. Potential Prebiotics for Improving Intestinal Diseases

2.3.1. Mouse Model

Mice (C57BL/6J, male, 9 weeks old at the time of purchase, 18 ± 2 g) were acquired from Orient Bio (Sungnam, Republic of Korea). The mice were held in a pathogen-free enclosure for 7 days prior to the exposure of DSS to some mice. For 5 days, 3% (*w*/*v*) of DSS was provided in drinking water, followed by 3 days with no DSS (molecular weight 36–50 kDa; MP Biomedicals, Irvine, CA, USA). During the whole experimental period of 8 days, CFE and sulfasalazine (St. Louis, MO, USA) were given to mice daily via oral administration. Thirty mice were divided randomly into six groups of five: (1) normal group fed water (N group), (2) negative control group fed DSS (NC group), (3) positive control group fed DSS + sulfasalazine (150 mg/kg) (PC group), (4) DSS + LCFE group fed DSS + 75 mg of CFE per kg of body weight, (5) DSS + MCFE group fed DSS + 150 mg of CFE per kg of body weight, and (6) DSS + HCFE group fed DSS + 300 mg of CFE per kg of body weight. The Animal Ethics Review Committee of Woojung Bio Inc. (Suwon, Korea) reviewed and approved these animal experiments in line with the Institutional Animal Care and Use Committee guidelines. The approval ID for using the animals at the Animal Facility of Woojung Bio was IACUC2303–041.

2.3.2. Evaluation of the Severity of Colitis

Colon length was measured, as in Zong et al. [32], from the ileocecal junction to the anal verge. We used the colitis DAI scoring system, as in Jeon et al. [33], to evaluate the severity of colitis in the examined mice.

2.3.3. Myeloperoxidase (MPO) Activity

The MPO assay kit (Abcam, Cambridge, MA, USA) was used to measure MPO activity in serum and tissue samples according to the manufacturer's instructions. Absorbance was read at 412 nm using a multimode microplate reader (BioTek Instruments, Winooski, VT, USA). The results were presented as units per gram of tissue.

2.3.4. Histological Evaluation

Intestinal tissue was fixed with 10% formalin, embedded in paraffin, cut into 3 µm sections, and stained with hematoxylin and eosin (H&E) for microscopic evaluation. The stained slices were subsequently observed under an optical microscope and analyzed using the i-Solution Lite software ver. 8.1 program (Innerview Co., Sungnam, Republic of Korea). Histological evaluations of H&E-stained colonic sections were graded by two blinded investigators (Table 3).

Table 3. Histological grading of colitis.

Grade	Infiltration Lesion	Epithelial Lesion
0	None	None
1	Infiltration around crypt bases	Some loss of goblet cells
2	Infiltration spreading to muscularis mucosa	Extensive loss of goblet cells
3	Extensive infiltration in the muscularis Mucosa with abundant edema	Some loss of crypt
4	Infiltration spreading to submucosa	Extensive loss of crypt

2.3.5. Statistical Analysis

The *t*-test, one-way ANOVA for comparison of two or more groups, and post-hoc Tukey's multiple comparison test were conducted using the SPSS 22.0 software (IBM Corp., Armonk, NY, USA). All data are presented as mean ± standard deviation.

3. Results

3.1. Effect of CFE on the Growth of Individual Bacteria

After feeding on CFE for 10 weeks, changes in the DNA log copy number of bacteria in mouse feces were determined (Figure 1). During CFE intake (10 weeks), an increase in beneficial bacteria, *A. muciniphila,* and *Bifidobacterium* spp., was observed, while the pathogenic bacteria, *S. aureus* and *Clostridium* spp., decreased. Moreover, in the HCEF group, *A. muciniphila* significantly increased while *S. aureus* significantly decreased (Figure 1a,c).

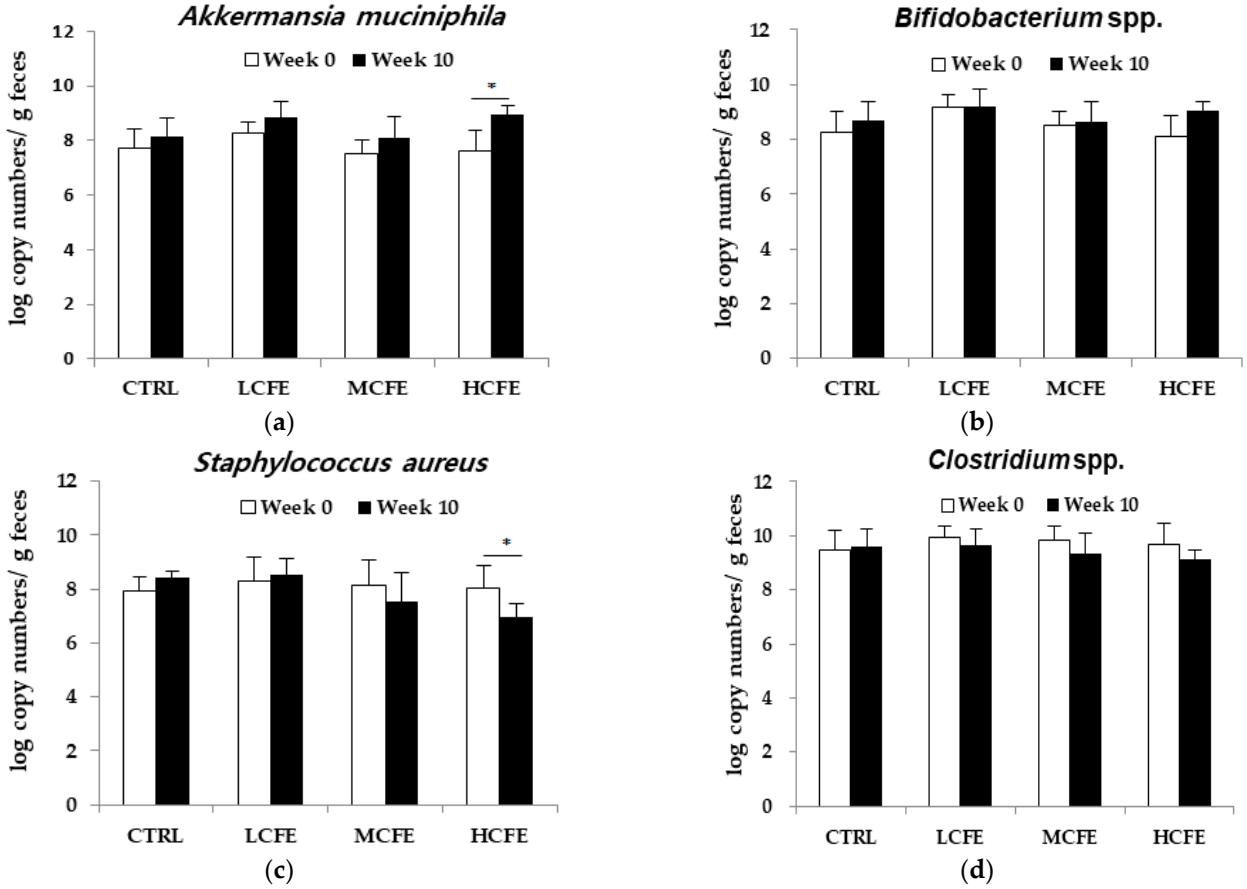

Figure 1. Changes in the number of *Akkermansia muciniphila* (**a**), *Bifidobacterium* spp. (**b**), *Staphylococcus aureus* (**c**), and *Clostridium* spp. (**d**) in the feces of mice fed CFE, as determined by quantitative PCR. The data shown are the mean ± SD of five independent experiments. Significant differences are noted as * $p < 0.05$ compared with week 0.

3.2. Effect of CFE on Cecum SCFA Production

As shown in Table 4, the main metabolite in mouse cecum contents was acetic acid, followed by butyric acid in small amounts. The contents of SCFAs in CFE-fed groups were higher than in the CTRL group in a CFE concentration-dependent manner. The content of acetic acid in the LCFE group ($p < 0.05$), the MCFE and HCFE groups ($p < 0.001$), and butyric acid in the MCFE and HCFE groups ($p < 0.05$) were higher than in the CTRL group.

Table 4. Analysis of SCFAs in mouse cecum contents.

Groups	SCFAs (μmol/g)	
	Acetic Acid	Butyric Acid
CTRL	9.05 ± 2.03	2.54 ± 0.48
LCFE	13.83 ± 1.12 **	3.27 ± 0.98
MCFE	18.91 ± 2.70 ***	3.70 ± 0.53 *
HCFE	20.24 ± 1.60 ***	3.94 ± 0.69 *

Values are mean ± SD of 5 mice. Significant differences are noted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the control group.

3.3. Effect of CFE on Fecal β-Glucuronidase Activities

β-Glucuronidase activities in mouse feces were measured at week 10 of CFE feeding (Figure 2). A marked decrease in β-glucuronidase activity was observed in the MCFE and HCFE groups ($p < 0.05$) compared with the CTRL group, being 88% and 87%, respectively.

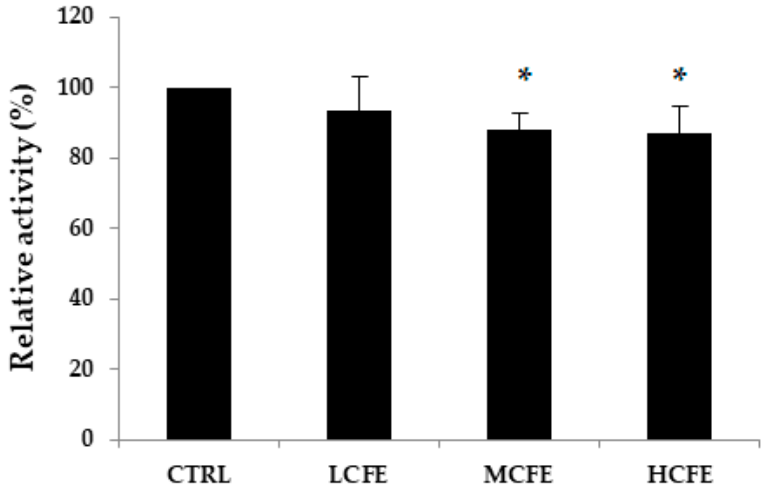

Figure 2. Relative fecal β-glucuronidase activity. The data shown are the mean ± SD of five independent experiments. Significant differences are noted as * $p < 0.05$ compared with the control group.

3.4. Effect of CFE on Mouse Colitis

To evaluate the effects of CFE on colitis, 9-week-old mice (C57BL/6) were administrated 3% DSS, sulfasalazine (150 mg/kg), and CFE (75 mg, 150 mg, and 300 mg per kg of body weight) separately or in combination, for 8 days (Figure 3a). The DAI score and colon length directly reflect the severity of UC in mouse models and are used to proactively assess the severity of UC. The DAI scores of each mouse group during days 0–7 are presented in Figure 3b. The DAI scores were significantly decreased in the CFE-fed group compared with the NC group. Another indicator that reflects the severity of intestinal inflammation is colon length, which recovers as inflammation improves [34,35]. The colon length of mice in the DSS + HCFE group was significantly longer ($p < 0.05$) than in mice in the NC group but similar to that of mice in the PC group (Figure 3c,d).

3.5. Effect of CFE on the Histological Injury of Colonic Epithelium Caused by DSS

H&E staining was performed to investigate mucosal inflammation. Compared with the N group, the DSS + HCHE (CFE-fed) group or the PC group showed lower microscopic damage. The histological analysis indicated that the histological severity of the colitis was more severe in the NC group compared with the CFE-fed and PC groups (Figure 4b). Compared with the NC group, the DSS + HCHE group showed a significantly decreased histology score. Test results showed that MPO activity in the colon was significantly reduced in the CFE-fed and PC groups compared with the NC group (Figure 4c).

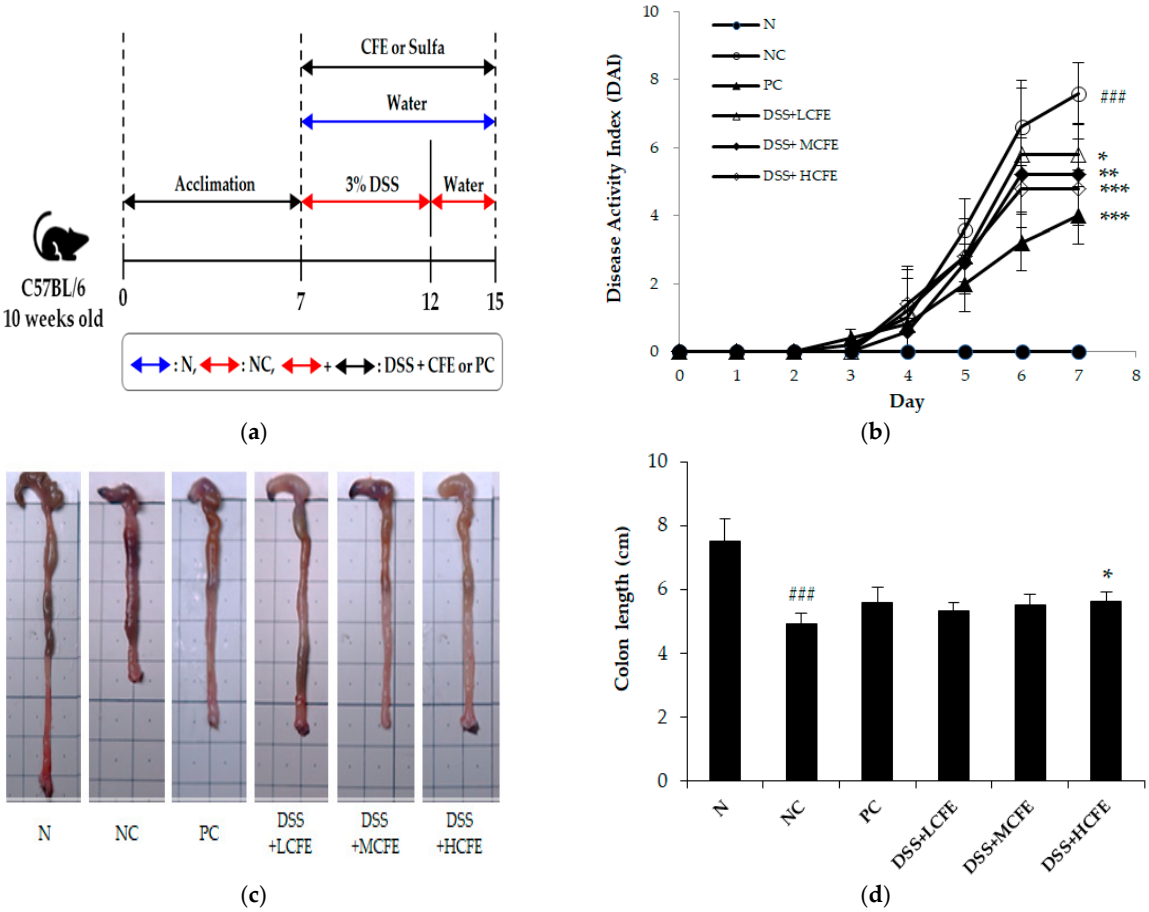

Figure 3. Effect of CFE on DSS-induced mouse colitis. (**a**) Experiment design, (**b**) DAI (disease activity index) score, (**c**) Macroscopic appearance, (**d**) Colon length of mice in each group. The data shown are the mean ± SD of five independent experiments. Significant differences are noted as $^{###}$ $p < 0.05$ compared with the normal group and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the negative control group.

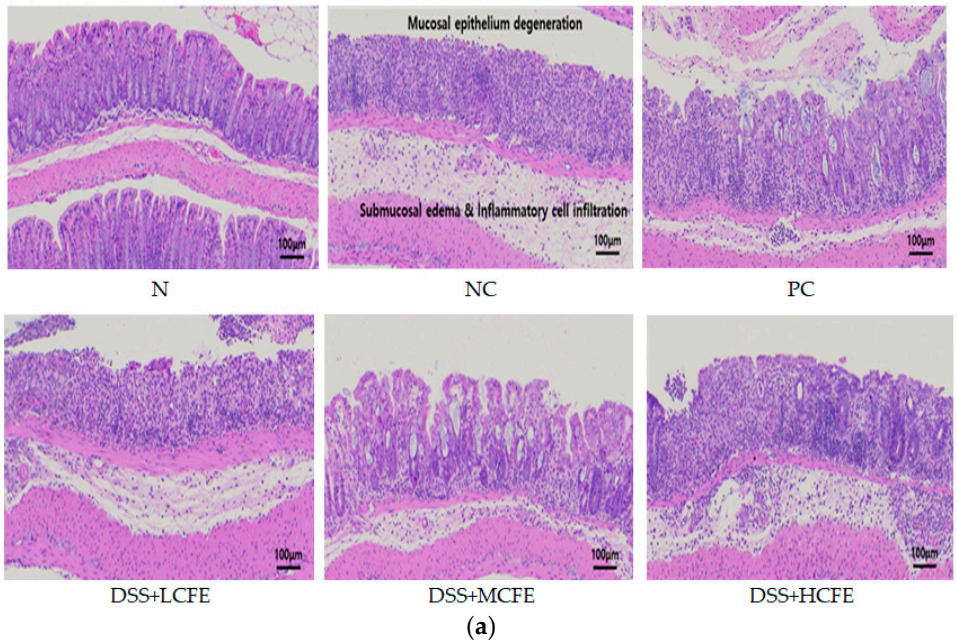

Figure 4. *Cont.*

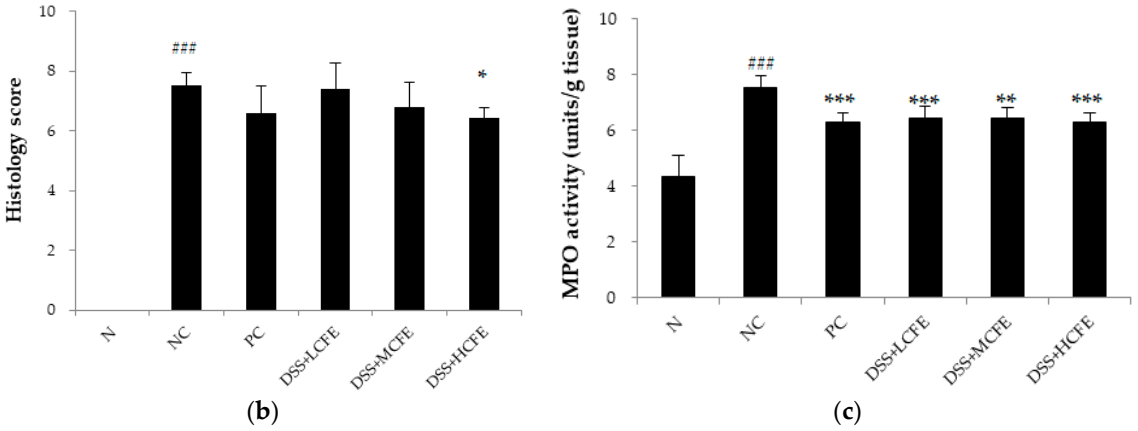

Figure 4. CFE prevented DSS-induced colon damage in mice. (**a**) H&E staining of colon, magnification ×100, (**b**) Histology score, (**c**) MPO (myeloperoxidase) activity. The data shown are the mean ± SD of five independent experiments. Significant differences are noted as $^{###}$ $p < 0.05$ compared with the normal group and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the negative control group.

4. Discussion

Sulfated polysaccharides derived from seaweed can be used as prebiotics for gut microorganisms and degraded into other bioactive compounds, such as oligosaccharides, phytochemicals, and SCFAs, which can serve as substrates for these organisms and allow them to grow [36–40]. Sulfated galactan from *C. fragile* consists of a large amount of galactose residues, with trace arabinose and the presence of pyruvate and sulfate as substituents [7]. Additionally, our previous research reported that CFE comprises many galactose residues, with traces of arabinose and the presence of sulfate as substituents [30].

In the present study, we elucidated the ability of CFE to promote the growth of *A. muciniphila*. The presence of *A. muciniphila* in feces has been associated with a healthy intestine, and its abundance has been inversely correlated with several disease states. Further analysis confirmed that *A. muciniphila* can degrade mucin and exert competitive inhibition on other pathogenic bacteria that degrade the mucin [41]. Therefore, the present study aimed to investigate the efficacy and underlying mechanisms of CFE in alleviating DSS-induced colitis in mice.

The proliferation of bacteria during 10 weeks of CFE feeding was determined using quantitative PCR. As the administration of CFE increased, the proliferation of probiotics increased whereas the proliferation of pathogenic bacteria decreased. Therefore, our results indicate that CFE can be used as a prebiotic material. Moreover, *A. muciniphila* significantly increased in the HCEF group. *A. muciniphila* is abundant in the gut microbiota of healthy individuals, and it is beneficial in the prevention and treatment of obesity, type 2 diabetes, and other metabolic dysfunctions [18–21,42–45]. Therefore, CFE can be used as a prebiotic that specifically promotes *A. muciniphila* growth for the treatment of metabolic diseases.

After CFE administration for 10 weeks, acetic acid was identified as the major metabolite in mouse cecum. A similar result was also reported by Li et al. [46], who found that acetic acid was a major metabolite produced by *A. muciniphila* in static and dynamic cultures. Li et al. [46] reported that acetic acid increased lipolysis and decreased lipid synthesis in BRL-3A cells, thus reducing the accumulation of hepatic fat in BRL-3A cells. Therefore, CFE was thought to reduce lipid synthesis as it led to the production of acetic acid by promoting the growth of *A. muciniphila.*

β-Glucuronidase activity is a major factor in causing colon cancer [47]. β-Glucuronidase hydrolyzes β-D-glucuronides to glucuronic acid and aglycone, such as alcohol, amine, imine, or a thiol compound. UDP-glucuronosyltransferase catalyzes glucuronide formation. From the liver, where synthesis occurs, it is partially eliminated into the large intestines along with bile. There, it is hydrolyzed to aglycone under the influence of bacterial β-glucuronidase, which is further hydrolyzed to aglycones. High β-glucuronidase activity

was observed in patients diagnosed with colonic neoplasia, suggesting that this enzyme plays an important role in promoting colonic neoplasia [48]. During the adaptation period, all mice were fed the AIN-93 diet for 1 week, but the enzymatic activity in each group at week 0 was not the same because the large intestines of all mice form a complex microbial ecosystem. A marked decrease in enzymatic activity was observed in the MCFE and HCFE groups compared with the CTRL group, being 88% and 87%, respectively. These results suggest that β-glucuronidase activity is decreased under CFE feeding of more than 150 mg per kg of body weight.

Dextran sulfate sodium (DSS)–induced colitis is mainly used to evaluate its efficacy against inflammatory bowel disease. Disease activity index (DAI) is increased, and colon length is shortened in DSS-induced colitis [49]. CFE treatment significantly improved the body weight, reduced the overall DAI score, and improved the colon length, suggesting the protective effect of CFE in DSS-induced colitis. Histologic examination consistently revealed an improvement in inflammatory signs, reducing inflammatory infiltrates and restoring intestinal epithelia in CFE-fed groups compared with the NC group, demonstrating that CFE alleviates mouse colitis.

MPO activity is a marker of neutrophil infiltration and is proportional to the number of neutrophils in the inflamed tissue [50]. In our study, MPO activity in the colon was significantly reduced in the CFE-fed group compared with the NC group, indicating that CFE can inhibit neutrophil infiltration and inflammation in mice.

5. Conclusions

In conclusion, the protective effect of CFE against DSS colitis suggests its clinical use by IBD patients. Further detailed studies would be needed to deepen *A. muciniphila* activity in relation to microecological interventions for IBD, and additional preclinical studies are needed to elucidate the underlying molecular mechanisms regulated by CFE in animal models of DSS-induced colitis. The conventional anti-inflammatory drugs used for treating IBD cause side effects such as allergic responses, diarrhea, vomiting, lymphopenia, raised liver enzymes, and inflammation of the pancreas [51]. It is important to alleviate IBD using natural plants with low toxicity and few side effects. Therefore, further studies on optimal dosage and safety in humans are needed for the development of natural products with enhanced properties for IBD prevention.

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Institutional Review Board Statement: Animal experiments were approved and performed in accordance with the guidelines of the Berry & Biofood Research Institute (BBRI-IACUC-21001) and Woojung Bio, Inc. (IACUC2303–041).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting the reported results can be provided by the corresponding author at reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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