Probiotics & Antimicro. Prot. (2017) 9:48–55
DOI 10.1007/s12602-016-9226-x

Protective Effects of *Mekabu* Aqueous Solution Fermented by *Lactobacillus plantarum* Sanriku-SU7 on Human Enterocyte-Like HT-29-luc Cells and DSS-Induced Murine IBD Model

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Published online: 17 August 2016

Abstract Most wakame *Undaria pinnatifida*, a brown algae, products are made from the frond portion. In this study, the polysaccharide content and antioxidant property of aqueous extract solutions (AESs) of the four parts (frond: *wakame*, stem of the frond: *kuki-wakame*, sporophyll: *mekabu*, and *kuki-mekabu*) of wakame were investigated. Polysaccharide content was high in both the *wakame* and *mekabu*. Superoxide anion (O_2^-) radical-scavenging capacities were high in the *mekabu*. These AESs could be fermented by *Lactobacillus plantarum* Sanriku-SU7. The O_2^- radical-scavenging activity of the *kuki-wakame, mekabu,* and *kuki-mekabu* were increased by the fermentation. Fermented *mekabu* clearly showed a protective effect on human enterocyte-like HT-29-luc cells and in a mouse model of dextran sodium sulphate-induced inflammatory bowel disease (IBD). These results suggest that the *mekabu* fermented by *L. plantarum* Sanriku-SU7 has anti-IBD effect related to O_2^- radical-scavenging.

Keywords *Lactobacillus plantarum* · *Undaria pinnatifida* · Superoxide anion radical-scavenging · Human enterocyte-like HT-29-luc cells · IBD modelled mouse

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Introduction

Since ancient times, inhabitants of the coastal regions of Far East countries, such as Korea and Japan, have collected algae from near the coast for consumption [1]. Wakame *Undaria pinnatifida* is one of most popular and important edible brown algae in these countries [2]. Wakame has become popular in other countries because it contains valuable nutritional and functional compounds, such as water-soluble dietary fibres, minerals, peptides, and phenolic compounds [3–5]. Their functional properties include antioxidant capacities, anti-glycation activity, cholesterol-lowering capacity, and improvement of the intestinal environment [3, 6–9].

The body of the wakame can be divided roughly into five parts: the frond, stem of frond, sporophyll, stem of sporophyll, and holdfast. The frond (*wakame*) is the most common wakame product that is boiled, salted, and dried [10]. About 300–400 thousand tons of these products are distributed in Japan. The sporophyll, called *mekabu*, is also a general algal food in Japan; its production is about 10 % of that of the boiled and salted wakame. Recently, there have been many reports on the functions of *mekabu* [11]. Part of the stem of *wakame*, called *kuki-wakame,* is used in delicacies; it is boiled and salted, but not dried. On the other hand, the entire stem of the *mekabu* and holdfast, called *ganiashi*, is not used. The Ministry of the Environment of the Government of Japan defines a *Satoumi* as a coastal area with increased biological productivity and biodiversity owing to human activity [12]. The traditional consumption of various algae is considered one of the features of the *Satoumi* region in Japan.

Reactive oxygen species, such as superoxide anion radicals ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen, are generated in body of breathing creatures. These oxygens react with DNA, proteins, lipids, and small cellular molecules and induce a wide range of common diseases and age-related degenerative conditions [13]. Correlation between the reactive oxygens and the age-related diseases, such as cardiovascular disease, inflammatory conditions, and neurodegenerative diseases such as Alzheimer's disease and cancer [14–17], had been reported. In our previous studies, some selected lactic acid bacteria (LAB) strains isolated from fish intestines and fermented fish showed antioxidant and anti-inflammatory properties in vitro and in vivo [6, 18–21]. A part of the LAB strains can ferment aqueous extract solution (AES) of some edible algae including wakame [7, 22].

In this study, to clarify the functional food properties of wakame and fermented wakame obtained from the Sanriku (Northeast coasts in Honshu Island, Japan) *Satoumi* region, we determined the in vitro antioxidant capacities and inhibitory effect on hydrogen peroxide toxicity in human enterocyte-like cells of the AESs and the fermented AESs with *Lactobacillus plantarum*. Then, effects of the *mekabu* and fermented *mekabu* AESs on mouse model of dextran sodium sulphate (DSS)-induced inflammatory bowel disease (IBD) were determined.

Materials and Methods

Preparation of Aqueous Extract Solutions from Wakame

Fresh wakame body was obtained from a culture bed in a small port in Shizugawa Bay, Miyagi, Japan (Fig. 1A) in March 2015. Usually, the frond (*wakame*), stem of frond (*kuki-wakame*), sporophyll (*mekabu*), and stem of sporophyll (*kuki-mekabu*) of the fresh wakame are separated (Fig. 1B). In the present study, the body of the fresh wakame was chilled in ice and transferred to our laboratory within 2 days. Each portion was then divided and milled using a blender (Oster 16 Speed Blender; Osaka Chemical Co., Osaka, Japan) with four times the volume of distilled water and heated at 105 °C for 15 min using an autoclave [23]. After cooling with tap water, the algae suspension was centrifuged at 2200 × *g* for 10 min at 4 °C. The collected supernatant was stored at −20 °C as the algal aqueous extract solution (AES).

Water-Soluble Polysaccharide Contents and Relative Viscosity of the AES

Water-soluble polysaccharide content in the AESs was measured by hot water extraction and an alcohol precipitation method that was reported previously [3]. The relative viscosity of the AESs was determined by an oscillation viscometer (Viscomate VM-1 G, Yamaichi Electronics, Osaka, Japan) under ice cooling. It was calculated as the quotient of the sample viscosity divided by the viscosity of distilled water [23].

Phenolic Content and Antioxidant Properties

Total phenolic content of the AES of wakame was determined using Folin-Ciocalteu solution as described previously [23]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion (O_2^-) radical-scavenging activities of the AES were determined using the colorimetric microplate assays as outlined in previous studies [24, 25]. The phenolic content and O_2^- radical-scavenging activity were evaluated as phloroglucinol equivalent (PGEq) and ascorbic acid equivalent (AAEq), respectively.

Fermentation by *Lactobacillus plantarum* Sanriku-SU7

In our previous study, a strain of *L. plantarum* Sanriku-SU7 (Accession No. LC122588) was isolated from a fermented fish product obtained from Iwate prefecture, Japan. Then, this strain was selected as a both *wakame* and *mekabu* fermentable strain. *L. plantarum* Sanriku-SU7 was precultured at 30 °C for 24 h with de Man, Rogosa, and Sharpe (MRS) broth (Oxoid; Basingstoke, UK). The pre-

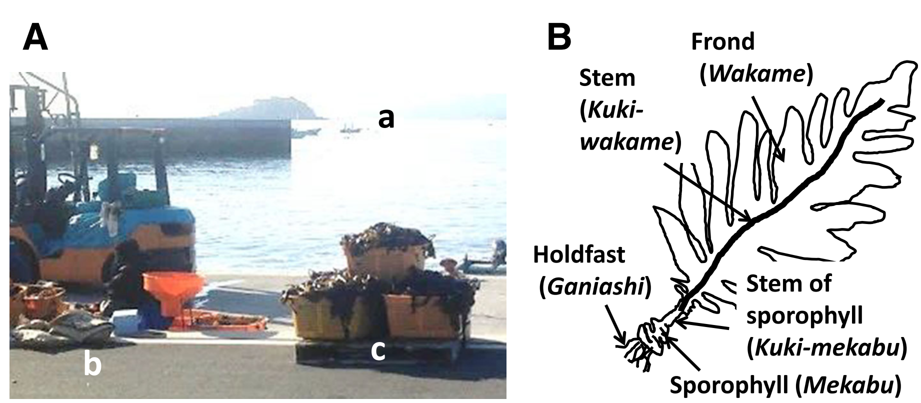

Fig. 1 Images of the harvest of wakame *U. pinnatifida* in the Shizugawa Bay, Miyagi, Japan (**A**) and names of wakame portions (**B**). *a* Cultivation area. *b* Cutting by hands. *c* Cut and divided wakame

Table 1 Polysaccharide and total phenolic compound contents and superoxide anion radical-scavenging activity of aqueous extract solution of *U. pinnatifida*

	Wakmae	Stem of *wakame*	*Mekabu*	Stem of *mekabu*
Relative viscosity (water = 1.0)	10.41 ± 0.36	4.15 ± 0.23	2.27 ± 0.02	1.18 ± 0.02
Polysaccharides (mg/ml)	11.10 ± 1.91	3.17 ± 0.80	11.10 ± 1.08	0.23 ± 0.13
Total phenolic content (μmol PGEq/ml)	14.56 ± 0.43	5.69 ± 0.96	21.71 ± 1.05	4.31 ± 1.05
O_2^- radical-scavenging (μmol AAEq/ml)	49.1 ± 4.5	7.4 ± 0.1	371 ± 13	13.3 ± 1.8

cultured strains (0.1 ml) were inoculated into 4 ml of the AES and incubated at 30 °C for 3 days. Turbidity was observed with the naked eye, and the pH value was determined using a pH meter (Twin pH; Horiba; Kyoto, Japan). DPPH and O_2^- radical-scavenging capacities were measured as described above.

Protective Effects Against Hydrogen Peroxide Toxicity on Human Enterocyte-Like HT-29-luc Cells

To determine the effects on the human enterocyte-like cells, the AES of *mekabu* and fermented *mekabu* was heated in boiling water for 20 min. HT-29-luc JCRB1383 was obtained from JCRB Cell Bank, National Institute of Biomedical Innovation (Ibaraki, Osaka, Japan). To induce differentiation, the cells were pre-incubated in a 48-well microplate with DMEM (Wako Pure Chemical, Osaka, Japan) containing 10 % (w/w) heat-inactivated FBS at 37 °C for 7 days under 5 % CO_2. Differentiation was confirmed by a colorimetric assay to determine the alkaline phosphatase activity in cells [26, 27].

The AES or fermented AES of wakame (0.06 ml) was added to the HT-29-luc culture (0.5 ml/well). After 4 h of incubation, 0.04 ml of H_2O_2 (final concentration, 3 mmol/L) was added. After 20 h of incubation, the survival rate was quantified by the neutral-red incorporation assay [19].

Protective Effect of *Mekabu* AES on Dextran Sodium Sulphate (DSS)-Induced IBD Mouse Model

The animal experiments were performed in compliance with the fundamental guidelines for proper conduct of animal experiments and related activities in academic research institutions, under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and approved by the animal experiment committee of the Tokyo University of Marine Science and Technology (Approval No. H27-4).

Anti-IBD effect was determined using DSS-induced murine IBD model as outlined in previous studies [6, 20]. Twenty-four 5-week-old male ddY mice were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). The mice were acclimated in a negative pressure rack maintained at 20–24 °C, with 50–60 % relative humidity and fed CE2 diet (CLEA Japan, Tokyo, Japan) and distilled water. After 5 days, the mice were divided into four groups ($n = 6$). Among them, two (DW and DW + DSS) groups were administered the same diet and distilled water. The other (M and FM) groups were administered the same diet but the 25 % AES of *mekabu* and fermented *mekabu*, respectively, instead of drinking water. After 3 days, 5 % (w/v) DSS (MW = 5000; Wako Pure Chemical, Osaka, Japan) was added to the drinking water of DW + DSS, M and FM groups. The mice received diet and water ad libitum. After 7 days of DSS administration, mice were anesthetised with diethyl ether and exsanguinated from the abdominal aorta. The large intestine (colon) was excised and washed with phosphate-buffered saline (PBS, Nissui Pharmaceuticals, Tokyo, Japan), and then the length was measured. The degree of IBD severity was also evaluated from histological observations based on haematoxylin and eosin (HE)-stained images of the colon [28]. Approximately 5-mm-long section of the middle part of the colon was soaked in 10 % formalin to prepare samples for the microscopic analysis and HE staining MedRidge (Tokyo, Japan).

Statistical Analysis

Data were presented as mean and standard error of the mean (SEM). Antioxidant capacities of the AES before and after fermentation were analysed by Student's *t* test. Data of the in vitro and in vivo experiments were subjected to ANOVA and Dunnett's post hoc tests using statistical software (Excel Statistic Ver. 6, Esumi, Tokyo, Japan).

Results and Discussion

Relative Viscosity and Water-Soluble Polysaccharides

As shown in Table 1, relative viscosity was the highest (about 10) in the *wakame* AES, followed by *kuki-wakame* and *mekabu* AESs (4.2 and 2.3, respectively). On the other hand, the water-soluble polysaccharide content was high in both the *wakame* and *mekabu*, at about 11 mg/ml. The content of the *kuki-wakame* AES was only 0.2 mg/ml.

Brown algae contain three water-soluble polysaccharides: alginic acid (polymer of urinic acids), laminaran (β-1,6 branched β-1,3 glucan), and fucoidan (sulfated fucans). The major viscous compounds are the alginic acids [23]. Although it can be seasonal, *mekabu* is rich in fucoidans [3]. There are many reports on the health-related functions of *mekabu* products containing fucoidans, particularly in regard to the immune system [29].

Total Phenolic Content and Antioxidant Properties

The phenolic concentration was the highest in the *mekabu* AES (22 μmol PGEq/ml), followed by the *wakame* AES (15 μmol PGEq/ml). The content in the *kuki-wakame* and *kuki-mekabu* was 6 and 4 μmol PGEq/ml, respectively. These values were not as high as the concentrations in the AES of other brown algal products shown in our previous reports, such as *Ecklonia stolonifera* and *E. kurome* [23].

Although DPPH has been used extensively as a free radical to evaluate reducing substances in various foods including edible algae [30], the capacity of the AES in this study was low and not clear (data are not shown).

In most organisms, O_2^- radicals are converted to hydrogen peroxide by superoxide dismutase. In the absence of transition metal ions, hydrogen peroxide is stable. However, hydroxyl radicals can be formed by the reaction of superoxide with hydrogen peroxide in the presence of metal ions, usually ferrous or copper [31]. Hydroxyl free radicals are more reactive (toxic) than superoxide anions. The capacity of the AESs to scavenge O_2^- radicals was confirmed when the radicals were generated by a chemical system comprising PMS, NADH, and oxygen. The O_2^- scavenging capacity of the *mekabu* AES was high (370 μmol AAEq/ml). On the other hand, the scavenging capacity of the other AESs, including *wakame*, was low.

In our previous report [7, 23], the correlation between DPPH radical-scavenging activity and phenolic content was high and the correlation between phenolic compound content and O_2^- radical-scavenging activity is not clear. It is considered that the O_2^- radical-scavenging activity of the AESs is dependent not only on the phenolic compounds but also on other water-soluble compounds such as peptides, polysaccharides, and Maillard reaction products [30, 32].

Effect of LAB Fermentation on O_2^- Radical-Scavenging Activity

Before fermentation, pH values of the AES of *wakame, kuki-wakame, mekabu,* and *kuki-mekabu* were 5.9, 6.1, 5.6, and 6.0, respectively. *L. plantarum* Sanriku-SU7 lowered the pH values to 3.9, 4.1, 3.9, and 4.3, respectively. No clear change was observed in the DPPH radical-scavenging activity. As shown in Fig. 2A, the O_2^- radical-scavenging activity of *mekabu* AES was increased by LAB fermentation. While the activity of *kuki-wakam*e and *kuki-mekabu* AES increased, they were not high. In the case of *wakame* AES, the radical-scavenging activity was lowered. Figure 2B, C shows the images of *mekabu* AES and fermented *mekabu* AES under phase-contrast microscopy. Before the fermentation, there were small fragments, about $10 \times 10\ \mu m^2$, in the AES, though it was centrifuged. After the fermentation, increasing and aggregation of *L. plantarum* Sanriku-SU7 and disappearance of the fragments of *mekabu* were shown.

Promotion of O_2^- radical-scavenging activity by LAB fermentation in white radish juice, milk, and soy milk has been reported [18, 21, 33]. It is thought that low-molecular-weight compounds including amino acids and lactic acid, generated during fermentation, and LAB cells have antioxidant potential [6, 22].

Inhibitory Effect of *Mekabu* AES on H_2O_2-Induced Damage in Human Enterocyte-Like Cells

Figure 3A shows the images of HT-29-luc cells exposed to 3 mmol/l H_2O_2 for 1 h and stained with neutral red. The confluent-attached cells were microscopically observed. H_2O_2 treatment detached 60 % of HT-29-luc cells (Fig. 3B). Fermented *mekabu* AES increased cell survival to about 70 %, higher than the seen with the non-fermented *mekabu*.

As mentioned above, hydroxyl radicals can be formed through the reaction of superoxide with H_2O_2 in the presence of metal ions such as ferrous and copper ions. Results of the inhibitory effect on the toxicity of H_2O_2 suggest that the fermented *mekabu* is beneficial for decreasing damage not only from H_2O_2 but also from hydroxyl radicals.

Protective Effect of Fermented *Mekabu* AES in DSS-Induced IBD Mouse Model

To determine the anti-inflammatory effect of *mekabu* AES and fermented *mekabu* AES in IBD, 5 % (w/v) DSS in drinking water was administered to mice with or without the AES. Five days after DSS administration, diarrhoea and bloody bowel discharge were observed in mice of DSS + DW and DSS + *mekabu* AES groups. In contrast, diarrhoea and bloody bowel discharge were not observed in mice administered fermented *mekabu* AES.

As shown in Fig. 4A, B, colon length was shorter in the mice administered DSS + DW compared to that observed for the control (DW without DSS) group mice. This represents the index of inflammation caused by IBD [28].

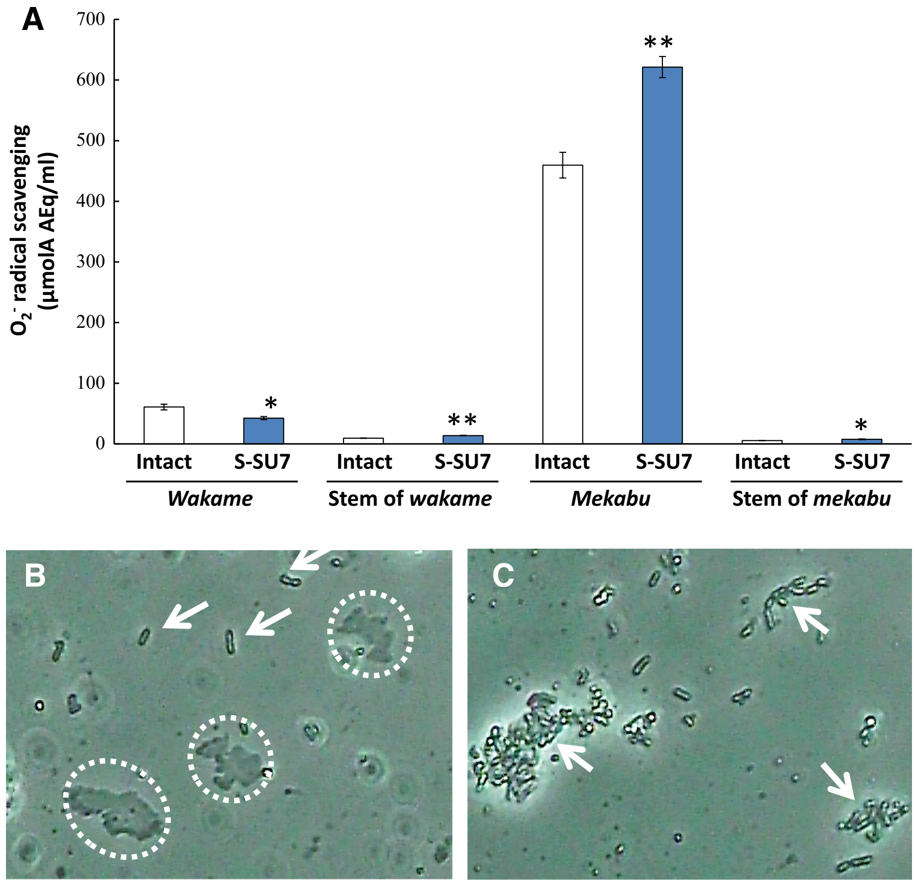

Fig. 2 Superoxide anion radical-scavenging activity of *wakame*, *kuki-wakame*, *mekabu*, and *kuki-mekabu* aqueous extract solutions (AESs, **A**) before (*open column*) and after (*closed column*) fermentation by *L. plantarum* Sanriku-SU7. Values are mean and SEM ($n = 3$). *Asterisks* are mean differences between *mekabu* and fermented *mekabu* AESs: $*p < 0.05$, $**p < 0.01$. **B**, **C** Results for AESs of *mekabu* before (**B**) and after (**C**) the fermentation. *Arrows L. plantarum* cells. *Circles* residue particles of *mekabu*

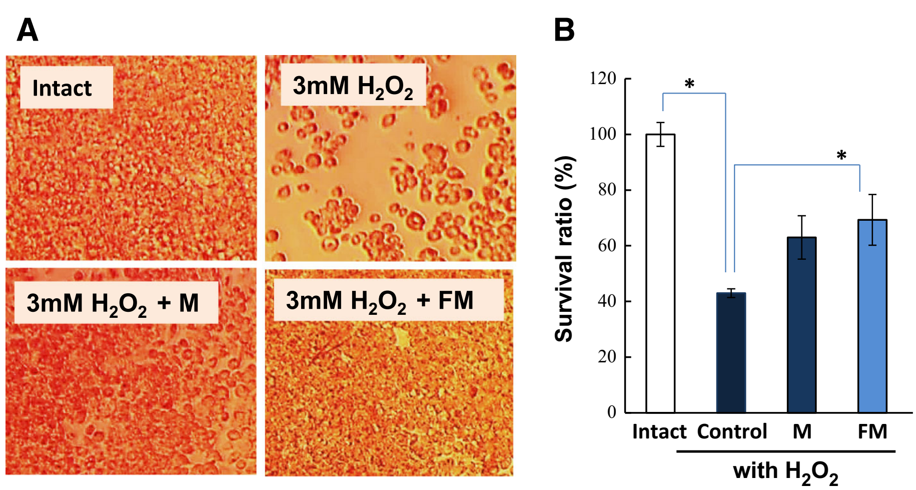

Fig. 3 Protective effect of aqueous solution of *mekabu* (M) and fermented *mekabu* (FM) against H_2O_2 toxicity in human enterocyte-like HT-29-luc cells. The cells were exposed to H_2O_2 for 20 h. After rinsing, neutral-red solution was added. **A** Images of cells stained with neutral red. **B** Ratio of absorbance at 550 nm. Values are mean and SEM ($n = 3$). *Asterisks* are mean differences compared to control: $*p < 0.05$

However, fermented *mekabu* AES recovered the colon length. This result indicates that fermented *mekabu* AES in drinking water significantly prevented IBD induced by DSS. Figure 4C shows typical images of HE-stained colon tissue. In the control group, the sections of the crypt structure in the mucosal layer, the submucosa, and muscular layer were normal. In the DSS control group, the crypt structure and submucosa were irregular. These irregularities caused by DSS were suppressed by fermented *wakame* AES. The results from this animal experiment were consistent with those of the in vitro experiments of O_2^- radical-scavenging activity (Fig. 2) and inhibitory effect on H_2O_2 toxicity performed using human enterocyte-like HT-29-luc cells (Fig. 3).

In this study, we determined the polysaccharide and phenolic contents, and antioxidant activity of AES of

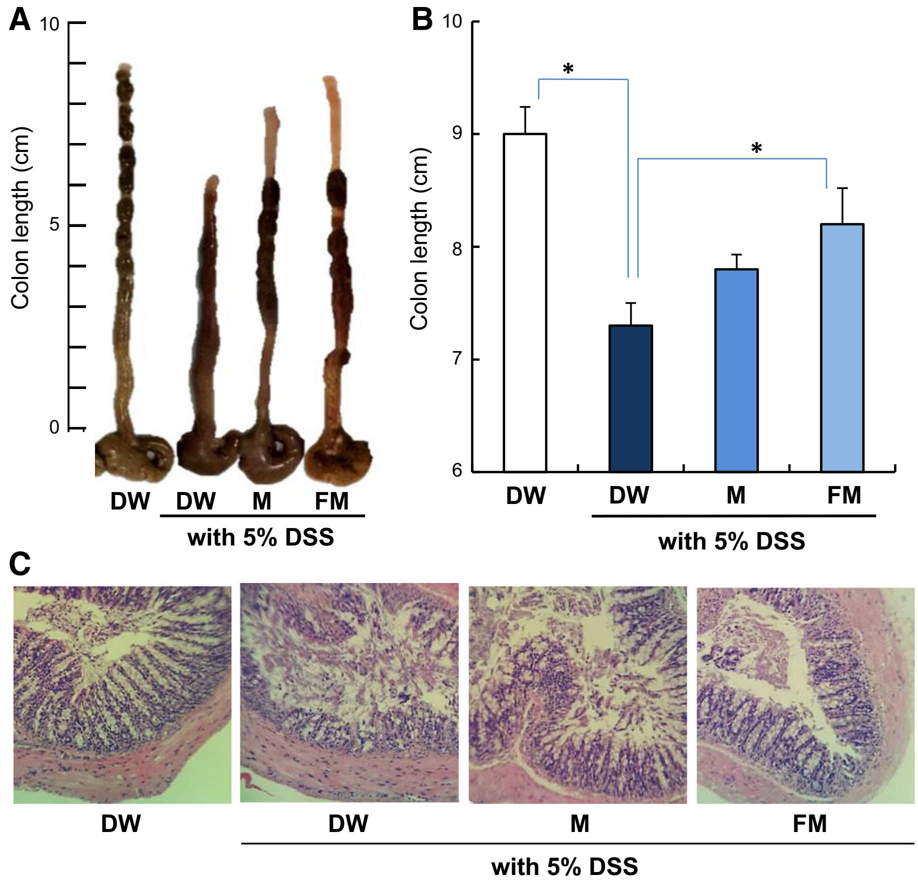

Fig. 4 Colon length of the mice (**A** and **B**) and images of HE-stained colon (**C**) of mice administered distilled water (DW), 5 % (w/v) DSS with DW, 5 % (w/v) DSS with 25 % aqueous extract solution (AES) of *mekabu* (M) or DSS with 25 % fermented *mekabu* AES (FM) in drinking water. Values in (**B**) are expressed as mean ± SEM ($n = 6$). *Asterisks* are mean differences compared to control: $*p < 0.05$

wakame *U. pinnatifida*. The water-soluble polysaccharide and phenolic contents and the O_2^- radical-scavenging activity were high in the AES of *mekabu*. The aqueous solutions can be fermented by *L. plantarum* Sanriku-SU7. In the fermented AES of *kuki-wakame, mekabu,* and *kuki-mekabu*, the in vitro O_2^- radical-scavenging activity increased. The fermented *mekabu* AES could protect human enterocyte-like HT-29-luc cells from reactive oxygen and improve colon condition in mice administered DSS. As mentioned above, reactive oxygen induces inflammatory diseases including IBD [34–36]. Therefore, foods having antioxidant capacity and compounds such as polyphenols were surveyed for their ameliorative effects in bowel diseases [37, 38]. Furthermore, there are several reports on the inhibitory effects of LAB [6, 39] and water-soluble saccharides including oligosaccharides and polysaccharides [40, 41]. Although the fermented *mekabu* AES was not rich in polyphenol compounds, it clearly showed a protective effect on human enterocyte-like cells and in IBD model mice. It can be suggested that some polysaccharides in *mekabu* and *L. plantarum* Sanriku-SU7 have a synergistic effect correlated with O_2^- radical-scavenging. Further studies to elucidate this effect and the mechanisms of *mekabu* compounds and *L. plantarum* Snriku-SU7 are needed.

Conclusion

In this study, the polysaccharide and total phenolic compound contents and antioxidant properties of the aqueous extract solution (AES) of four parts of wakame were determined. Polysaccharide content was high in both *wakame* and *mekabu*, although the relative viscosity of *mekabu* was not as high as *wakame*. Superoxide anion (O_2^-) radical-scavenging activity of the *mekabu* AES was high. These aqueous solutions could be fermented by *L. plantarum* Sanriku-SU7. The fermented AES of *kuki-wakame, mekabu,* and *kuki-mekabu* has increased O_2^- radical-scavenging activity in vitro. Although the fermented *mekabu* AES was not rich in polyphenol compounds, it clearly showed a protective effect on human enterocyte-like cells and in DSS-induced murine IBD model. It can be suggested that some polysaccharides in *mekabu* and *L. plantarum* Sanriku-SU7 have a synergistic effect correlated with O_2^- radical-scavenging.

Acknowledgments This work was supported by the SANRIKU Fisheries Research and Education Project (Iwate University, Ministry of Education, Culture, Sports, Science and Education and Technology [MEXT]) in Japan, The Towa Foundation for Food Science and Research, Tokyo, Japan, and TERRADA Warehouse, Tokyo, Japan.

Compliance with Ethical Standards

Conflict of interest Maki Nemoto, Takashi Kuda, Mika Eda, Hiroshi Yamakawa, Hajime Takahashi, and Bon Kimura declare that they have no conflict of interest.

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