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Gut health benefits of brown seaweed *Ecklonia radiata* and its polysaccharides demonstrated *in vivo* in a rat model

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ABSTRACT

We examined the gut health potential of the brown seaweed *Ecklonia radiata* and its polysaccharides. Rats consumed an AIN-93G-based diet without addition (control) or containing 5% (w/w) of ground dried whole seaweed (WS) or dried powdered polysaccharide fraction (PF) of the seaweed for 1 wk. The PF consisted largely of fucoidan and alginate. PF treatment increased cecal digesta weight relative to the control (1.36 ± 0.17 vs 0.60 ± 0.06 g/100 g body weight). Beneficial cecal total short chain fatty acids increased in response to WS (213.25 ± 14.40 μmol) and PF (208.59 ± 23.32 μmol) compared with the control (159.96 ± 13.10 μmol). Toxic protein fermentation product levels were decreased by WS and PF. Cecal numbers of bacteria relevant to gut health were determined using quantitative real-time PCR. Relative to the control, numbers of butyrate-producing *Faecalibacterium prausnitzii* were increased by PF supplementation, whereas WS decreased numbers of potentially pathogenic *Enterococcus*. In conclusion, *E. radiata*-derived polysaccharides have promise as prebiotic supplements.

1. Introduction

Prebiotics are defined as substrates that improve the host gut health by selectively stimulating the growth and/or metabolic activity of beneficial gut microbes (Roberfroid et al., 2010). Numerous varieties of indigestible oligo- and polysaccharides have been demonstrated as potential sources of dietary fibre and prebiotics (Praznik, Loeppert, Viernstein, Haslberger, & Unger, 2015). They can act as substrates for fermentation in the large bowel, leading to the production of short chain fatty acids (SCFA) with multiple functions that help maintain gut health (Conlon & Bird, 2015). Apart from polysaccharides, ingested polyphenols with complex structures can also reach the large intestine where they can be converted into beneficial bioactive metabolites by gut microbes (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013). Thus, there is a growing interest in isolating polysaccharides and polyphenols from novel sources such as marine seaweeds for use as prebiotics and gastrointestinal modification (Corona et al., 2016; de Jesus Raposo, de Morais, & de Morais, 2016; O'Sullivan et al., 2010).

A brown seaweed (*Ecklonia radiata*) commonly found along the coast of Southern Australia contains many nutrients which may benefit gut health, especially polysaccharides, which account for around 70% of its dry weight, and polyphenols, namely phlorotannins, that represent up to 6.5% of the dry weight (Charoensiddhi, Franco, Su, & Zhang, 2015). Our previous studies (Charoensiddhi, Conlon, Vuaran, Franco, & Zhang, 2016) demonstrated the prebiotic potential of extracts of this seaweed *in vitro*. When added to an *in vitro* anaerobic fermentation system containing human faecal inocula, the extracted components underwent fermentation, increased the production of SCFA, and promoted the growth of specific beneficial gut microbes. To understand the prebiotic potential of these seaweed-derived components further, we isolated a fraction from this seaweed which was enriched in non-digestible complex polysaccharides. This fraction subsequently stimulated the production of beneficial fermentation products by

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gut microbes, particularly butyric acid, when added to the *in vitro* anaerobic fermentation system (Charoensiddhi, Conlon, Vuaran, Franco, & Zhang, 2017). The prebiotic potential of extracts and fermentable components from other seaweeds such as *Osmundea pinnatifida*, *Laminaria digitata*, and *Enteromorpha prolifera* were also demonstrated *in vitro* using fermentation systems which mimic the human gut (Kong, Dong, Gao, & Jiang, 2016; Li et al., 2016; Michel, Lahaye, Bonnet, Mabeau, & Barry, 1996; Ramnani et al., 2012; Rodrigues et al., 2016).

The complex interactions between dietary components, gastrointestinal physiological processes, and gut microbiota are difficult to model *in vitro*. Consequently it is important to follow up promising *in vitro* results with *in vivo* testing. Therefore, the aim of the present study in rats was to understand whether the consumption of the brown seaweed *E. radiata* and a derived polysaccharide fraction will lead to beneficial changes in gut health. The production of SCFA and other fermentation products, and the impacts on the growth of selected microbes with potentially beneficial or pathogenic effects were used as indicators of prebiotic potential. Effects on digesta bulk were also examined, as increased stool bulk in humans is an important means of reducing colorectal disease, at least partly due to dilution of toxins. Finally, alginate and fucoidan were selectively extracted from the polysaccharide fraction and characterised in order to confirm their presence and effect contributing to the prebiotic activities.

2. Materials and methods

2.1. Preparation of the whole seaweed (WS)

Brown seaweed (*Ecklonia radiata*- identified by the State Herbarium of South Australia) was collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2016. It was rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. All seaweed materials were collected at one day to provide consistent samples for all studies. They were dry milled (Foss Cyclotec™ 1093, Hillerod, Denmark), then passed through a sieve, and dried in an oven at 45 °C to obtain a moisture content of approximately 10%. The WS was stored at −20 °C prior to extraction and supplementation in the rat diet.

2.2. Chemicals and substrates

All chemicals used are of analytical or chromatography grade from Merck and Sigma. A commercial carbohydrate hydrolytic enzyme (Viscozyme® L) used for the preparation of seaweed polysaccharide fraction (PF) was kindly provided by Novozymes (Bagsvaerd, Denmark).

2.3. Preparation of the PF

The PF was prepared according to the method of Charoensiddhi et al. (2017) with some modifications. Briefly, the WS was firstly extracted with 90% (v/v) ethanol at a seaweed solid to solvent ratio of 1:10 (w/v) to remove phlorotannins from the seaweed biomass. The suspension was incubated at room temperature for 3 h under continuous stirring at 80 rpm, and then centrifuged at 7350*g* for 10 min at 4 °C. The residue was dried and further dispersed in water with a ratio 1:10 (w/v). The pH was adjusted using 1 N HCl to achieve the optimum pH of Viscozyme at 4.5. The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed under an optimal condition at 50 °C for 3 h in a jacketed electric kettles VEL20 (Vulcan, Baltimore, MD, USA). The enzyme was inactivated by boiling the solution at 100 °C for 10 min and cooling with a cooling coil. The extract was centrifuged at 7350*g* for 10 min at 4 °C. The supernatant was collected and adjusted to pH 7.0 using 1 N NaOH. Ethanol was then added to the supernatant to a concentration of 67% (v/v) to precipitate the PF. The PF was left to precipitate at 4 °C overnight, then centrifuged at 7350*g* for 10 min at 4 °C, freeze dried, and stored at −20 °C until further use in the supplementation of the rat diet.

2.4. Analyses of the composition of WS and PF

The compositions of WS and PF were investigated according to the methods described in Charoensiddhi et al. (2016). Total protein, starch, dietary fibre, and non-digestible non-starch polysaccharide (NNSP) were determined using established AOAC methods. Total phlorotannin was analysed by Folin Ciocalteu's phenol reagent, and the results were expressed as g phloroglucinol equivalent. All results were expressed as g/100 g dry samples.

2.5. Animal experiments and diets

All experimental protocols related to animal experiments were approved by the Animal Ethics Committee of CSIRO Food and Nutritional Sciences and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty-four male Sprague-Dawley rats of ~200 g weight were obtained from the Animal Resource Centre, Murdoch University, Perth, Australia. They were housed in wire-bottomed cages (4 rats per cage) in a room with controlled temperature at 23 °C and lighting (a 12-h-light/-dark cycle) throughout the study. Rats were acclimatised for 1 wk following arrival, with free access to a standard commercial rat diet (Specialty Feeds, WA, Australia) and water. After acclimatisation, they were weighed, ear tagged, and assigned randomly to 3 groups (n = 8/group). All rats were given free access to water and powdered AIN-93G based diets (Reeves, Nielsen, & Fahey, 1993) with or without supplementation with 5% (w/w) of WS *E. radiata* or PF of the seaweed by reducing levels of cornstarch in the diet (Table 1) for 1 wk. Dietary intake was monitored every day, and the body weight for each animal was weighed on day 3 and 7 during the week of the experimental dietary treatment.

2.6. Sampling procedures and analysis methods

At the completion of the 1 wk dietary intervention period, fresh faeces were collected for moisture content analysis. Rats were then

Table 1
Compositions of the experimental diets used in the animal studies.

Ingredient[a]	Control diet	WS diet	PF diet
Casein	200	200	200
Cornstarch	530	480	480
Sucrose	100	100	100
WS	–	50	–
PF	–	–	50
Sunflower oil	70	70	70
Wheat bran	50	50	50
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Vitamins (AIN 93)	10	10	10
Minerals (AIN 93)	35	35	35
Tert-butyl hydroquinol	0.014	0.014	0.014

[a] All ingredients were reported based on g per 1000.514 g diet, and the suppliers of these diet ingredients were provided: Casein (Devondale Murray Goulburn, VIC, Australia); Cornstarch (PFD Foods, VIC, Australia); Sucrose (PFD Foods, VIC, Australia); Sunflower Oil (Woolworths, SA, Australia); Wheat bran (Woolworths, SA, Australia); L-Cystine, Choline bitartrate, and Tert-butyl hydroquinol (Sigma-Aldrich, St. Louis, MO, USA); Vitamins and minerals (ICN, Costa Mesa, CA, USA).

anesthetized with 4% isofluorane/oxygen and killed to allow the collection and weighing of gut tissues, digesta, and other organs (liver, kidney, spleen, thymus, small intestine, colon, and cecum). The lengths of small intestine and colon were also measured. Cecal digesta was frozen and stored at −80 °C for subsequent analyses of SCFA, phenols and *p*-cresols, and bacterial populations.

2.6.1. SCFA, phenol, and p-cresol analysis

SCFA were analysed using GC according to the modified method from Vreman, Dowling, Raubach, and Weiner (1978), and phenol and *p*-cresol were analysed using HPLC according to the modified methods from De Smet et al. (1998), Murray and Adams (1988) and King, May, Davies, and Bird (2009). Briefly, frozen cecal digesta was mixed thoroughly with internal standard solution (5.04 μmol/g cecal digesta of heptanoic acid and 150 μg/g cecal digesta of *o*-cresol). Samples were centrifuged at 2000*g*, 4 °C for 10 min, and 300 μL of supernatant was transferred to a pre-cooled tube. Then 100 μL of 10% (w/v) sulfosalicylic acid and 4 mL of ether were added and centrifuged at 2000*g*, 4 °C for 2 min. The ether top layer was transferred to a clean tube containing 200 μL of 0.05 M sodium hydroxide in methanol and mixed. The solvents were evaporated under a stream of nitrogen at room temperature, and 100 μL of water was added and mixed to dissolve the dried residue. 30 μL of 1 M phosphoric acid was added to the tube and immediately transferred to a cold GC vial and capped. Samples were loaded onto the GC (model 6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionisation detector and capillary column (Zebron ZB-FFAP, 30 m × 0.53 mm × 1.0 μm, Phenomenex, Lane Cove, NSW, Australia). Helium was used as the carrier gas; the initial oven temperature was 90 °C held for 1 min and increased at 20 °C/min to 190 °C held for 2.5 min; the injector and detector temperature was 210 °C; the gas flow and septum purge rates were at 7.7 and 3.0 mL/min, respectively. A standard SCFA mixture containing acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acids was used for the calculation. The fatty acid concentrations were calculated in μmol/g cecal digesta by comparing their peak areas with the standards, and total fatty acid amounts in the cecal digesta were reported by taking the weight of the cecal digesta into account.

After the GC analysis, the vials were transferred to the HPLC (LC-10 system; Shimadzu, Kyoto, Japan) equipped with RF-10AXL fluorescence detector and C18 column (Microsorb-MV 100-5, 250 mm × 4.6 mm, Agilent Technologies, Santa Clara, CA, USA) for analysis of phenol and *p*-cresol. Mobile phase consisted of 30% (v/v) acetonitrile, pH 3.2 at a flow rate of 1 mL/min. Phenol and *p*-cresol were calculated in μg/g cecal digesta by comparing their peak areas with the standards, and total phenol and *p*-cresol amounts in the cecal digesta were reported by taking the weight of the cecal digesta into account.

2.6.2. Microbial population enumeration

DNA was extracted from cecal digesta using bead beating followed by the PowerMag® Microbiome RNA/DNA Isolation Kit (27500-4-EP; MO BIO Laboratories, Inc., Carlsbad, CA, USA) optimised for epMotion (Charoensiddhi et al., 2016, Charoensiddhi et al., 2017). The extracted DNA was further purified by removal of protein and polysaccharide contaminants to obtain a high quality of nucleic acids following to the method of Greco, Sáez, Brown, and Bitonti (2014). The bacterial numbers were determined by quantitative real-time PCR (Q-PCR) with a series of microbe-specific primer pairs according to the methods described in Charoensiddhi et al. (2016) and Charoensiddhi et al. (2017).

2.7. Characterisation of the PF

2.7.1. Selective isolation of alginate and fucoidan

Alginate and fucoidans were selectively isolated from the PF in order to determine their levels (%w/w) and characteristics. The separation of alginate was carried out using the method described by McHugh (2003) and Sellimi et al. (2015). Briefly, the PF was dissolved in deionised water and adjusted to pH 8 with 2 M NaOH. 2 M $CaCl_2$ solution was slowly added with stirring until no further precipitation was observed, followed by centrifugation at 9000*g*, 25 °C for 15 min. After that $CaCl_2$ was added to the supernatant, and the suspension was centrifuged again to ensure that all of the calcium alginate had been recovered. The precipitated calcium alginate was collected for further purification, by suspending it in deionised water and reducing the pH to below 3 with the addition of 6 M HCl. The alginic acid precipitate was resuspended in deionised water, and 2 M NaOH was added to achieve a pH of 8. Sodium alginate derived from this process was then dialysed (Molecular weight (MW) cut off 1 kDa, Spectra/Por®7, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and freeze dried. Meanwhile, ethanol was added to the supernatant to 67% (v/v), and left overnight at 4 °C for complete precipitation. The precipitate was separated by centrifugation at 9000*g*, 25 °C for 15 min, and then dissolved in deionised water. Contaminants were removed using dialysis membrane (1 kDa) and the retentate was freeze dried to obtain fucoidan fraction.

2.7.2. MW analysis of alginate and fucoidan

High performance gel permeation chromatography (HPGPC, Waters 600E, Milford, MA, USA) were carried out at 30 °C using an Ultrahydrogel linear column (MW resolving range of 1000–20,000,000) with a guard column and a refractive index detector. Samples were eluted at a flow rate of 0.6 mL/min with 0.05 M sodium bicarbonate buffer (pH 11) as a mobile phase. The relative MWs of the samples were estimated using a calibration curve established with standard dextrans from Sigma (MWs of 4400–401,000 Da).

2.7.3. FT-IR spectroscopy

The infrared spectra of alginate and fucoidan were recorded using Spectrum One FT-IR Spectrometer (Perkin Elmer, Shelton, CT, USA) equipped with a MIR TGS detector. The samples were ground together with potassium bromide (KBr), and the mixture was transferred to the compression die and pressed into a pellet under high pressure. A region from 400 to 4000 cm^{-1} was used for scanning at 4 cm^{-1} resolution over 16 scans.

2.7.4. NMR analysis

Alginate and fucoidan (75 mg) were dissolved in 1 mL of D_2O (99.8% D). ^1H NMR and ^{13}C NMR were recorded at 20 °C on an AVANCE^{III} 500 MHz Digital NMR Spectrometer (Bruker Biospin AV-500, Rheinstetten, Germany). ^1H NMR spectra were recorded at a frequency of 500 MHz. The spectral parameters used were as follows: 1024 numbers of scan (NS), relaxation delay of 2 s, spin rate of 25 Hz, and spectral size 16 K with 32 K time domain size. ^{13}C NMR spectra were recorded at a frequency of 125 MHz. The spectral parameters used were as follows: 80,000 numbers of scan (NS), relaxation delay of 1 s, spin rate of 25 Hz and spectral size 32 K with 32 K time domain size.

2.7.5. Analysis of sulphate content

Sulphur was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES) using the model Activa Horiba Jobin Yvon (Kyoto, Japan). Prior to analysis, 150 mg of samples

containing a mixture of 2 mL sulphuric acid 72% (w/w) and 6 mL nitric acid 65% (w/w) were digested using a microwave system at 200 °C for 20 min (ramp-up time is 10 min). Calibration standards were prepared with sulphur concentrations of 0, 2.5, 5.0, 7.5, and 10 mg/L. The sulphate content was deduced from the amount of sulphur determined by ICP using the following equation: %Sulphate content = 3.22 × S%. Where 3.22 is the conversion factor for the sulphur to sulphate content (as the sodium salt), and S% is the percentage of sulphur in the samples (Rioux, Turgeon, & Beaulieu, 2007).

2.8. Statistical analysis

Data on the body, organ, and digesta weight/length and % fecal moisture were presented as the mean ± SEM for each treatment group (n = 8). Results of SCFA, phenol, and *p*-cresol analyses were expressed as mean ± SEM, n = 8 from analytical duplicate analyses. For bacterial enumeration, results were mean ± SEM, n = 8 from which DNA was extracted in duplicate. PCR amplification was carried out in triplicate from each of these DNA extracts. The effect of treatments was determined by ANOVA and differences between treatments were analysed post hoc by Tukey's test and considered significant at $P < 0.05$ using IBM SPSS Statistics 22 (IBM Corporation Software Group, Somers, NY, USA).

3. Results

3.1. Composition of the WS and PF

The compositions of key fermentable components in the WS and PF are shown in Table 2. Dietary fibre is the major component in the WS and PF. The PF contains higher dietary fibre and NNSP contents, while the WS contains higher levels of protein and phlorotannin. Starch was not detected in both WS and PF.

3.2. Effect of different diets on the body and organ weights

All results are summarised in Table 3. There were no significant differences in the final body weight between the control (AIN-93G based diet) and two treatment groups (the diets supplemented with 5% WS or PF), but the weight gain (%) observed from the start to the end of experiment for rats fed with WS diet (23%) was increased significantly ($P < 0.05$) when compared to other groups (~17%). Food intake measured on a daily basis was not different between any of the groups (data not shown). Although dietary treatments had no significant effects on the weight and the ratio of body weight to liver, kidney, spleen, thymus, and small intestine, there were significant impacts on cecum and colon. Cecal tissue and digesta weights (including the ratio to body weight) of rats fed with PF diet were significantly ($P < 0.05$) higher (1.4-fold for tissue; 2.3-fold for digesta) than the control and higher (1.3-fold for tissue; 1.5-fold for digesta) than the WS fed group. Although the weight of colon digesta was not significantly affected by the dietary treatments, the weight of colonic tissue in rats fed with PF diet was significantly ($P < 0.05$) higher (1.2-fold) than the control group. The colon length in two treatment groups fed with WS and PF diets was significantly ($P < 0.05$) longer (1.1-fold) than the control. The PF diet significantly ($P < 0.05$) increased fecal moisture of rats by 6% and 11% relative to WS and control diet groups, respectively.

Table 2
Main nutrients and potential fermentable components (g/100 g dried seaweed or fraction) of the WS and PF; values are means ± SEM of triplicate analyses (n = 3).

Composition (g/100 g)	WS	PF
Dietary fibre	59.6 ± 0.15	69.5 ± 0.30
NNSP	11.3 ± 0.84	17.6 ± 0.35
Starch	0	0
Protein	7.1 ± 0.03	5.6 ± 0.08
Phlorotannin	4.5 ± 0.10	1.4 ± 0.02

Table 3
Effect of dietary supplementation with WS and PF on the final body weight, gut tissue and digesta, organ weight/length, and fecal moisture; values are mean ± SEM, n = 8. Means in a row with different superscripts indicate significant differences ($P < 0.05$).

	Control	WS diet	PF diet
Initial body weight (g)	263.38 ± 3.25	261.13 ± 3.20	261.25 ± 2.74
Final body weight (g)	308.38 ± 5.82	321.75 ± 6.06	307.25 ± 4.51
Body weight gain[a] (%, 7 days)	17.09^{b} ± 1.67	23.17^{a} ± 1.16	17.65^{b} ± 1.67
Cecum weight (g)			
Tissue	0.70^{b} ± 0.03	0.76^{b} ± 0.03	0.99^{a} ± 0.03
Digesta	1.86^{b} ± 0.19	2.87^{b} ± 0.16	4.15^{a} ± 0.50
% Cecum weight (g/100 g body weight)			
Tissue	0.23^{b} ± 0.01	0.24^{b} ± 0.01	0.32^{a} ± 0.01
Digesta	0.60^{b} ± 0.06	0.89^{b} ± 0.05	1.36^{a} ± 0.17
Colon weight (g)			
Tissue	1.35^{b} ± 0.08	1.59^{a,b} ± 0.05	1.68^{a} ± 0.08
Digesta	0.67 ± 0.16	1.23 ± 0.28	1.15 ± 0.23
% Colon weight (g/100 g body weight)			
Tissue	0.44^{b} ± 0.02	0.49^{a,b} ± 0.01	0.55^{a} ± 0.02
Digesta	0.22 ± 0.05	0.38 ± 0.09	0.37 ± 0.07
Organ weight (g)			
Liver	14.16 ± 0.40	15.43 ± 0.35	14.30 ± 0.69
Kidney	1.13 ± 0.02	1.21 ± 0.04	1.16 ± 0.03
Spleen	0.69 ± 0.03	0.65 ± 0.01	0.69 ± 0.06
Thymus	0.52 ± 0.04	0.56 ± 0.04	0.55 ± 0.02
Small intestine	6.95 ± 0.22	7.27 ± 0.27	7.19 ± 0.22
% Organ weight (g/100 g body weight)			
Liver	4.59 ± 0.08	4.79 ± 0.06	4.64 ± 0.17
Kidney	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
Spleen	0.23 ± 0.01	0.20 ± 0.01	0.22 ± 0.02
Thymus	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
Small intestine	2.25 ± 0.05	2.26 ± 0.07	2.34 ± 0.05
Organ length (cm)			
Small intestine	128.13 ± 3.36	124.00 ± 2.59	129.75 ± 3.36
Colon	20.31^{b} ± 0.19	22.38^{a} ± 0.43	22.38^{a} ± 0.40
% Fecal moisture	59.78^{c} ± 0.42	62.91^{b} ± 0.36	66.78^{a} ± 0.35

[a] % Body weight gain was calculated based on (Final body weight − Initial body weight)/Initial body weight × 100.

3.3. SCFA, phenol, and p-cresol production

The cecal SCFA, phenol, and *p*-cresol production in rat cecal digesta are presented in Table 4. Total rat cecal SCFA (μmoL) produced from both WS and PF diets was significantly ($P < 0.05$) higher, approximately 20%, than that of the control group. Relative to the control group, the levels of acetic and propionic acids in the total rat cecal digesta of the rats fed with WS and PF diets were significantly higher at approximately 25–37%, while the highest level of butyric acid (increase of 20%) was observed in the rat cecal digesta of the PF diet group. Lower levels of iso-butyric, valeric, iso-valeric, and caproic acids were recorded for two treatment diet groups when compared to the control, and the pH of rat cecal digesta of the WS (7.04 ± 0.04) and PF (6.58 ± 0.09) diets significantly ($P < 0.05$) decreased in conjunction with SCFA production when compared to the control (7.68 ± 0.07). However, the concentrations of almost all individual and total SCFA (μmol/g cecal digesta) in rats fed with WS and PF diets were lower when compared to the control due to taking the weight of the cecal digesta into account. In contrast, the level and concentration of cecal phenol in rats fed with WS and PF diets were significantly ($P < 0.05$) lower than the control rats, approximately decreased up to 80%, with WS having a more profound effect. Similar results were

Table 4
Effect of WS and PF diets on level and concentration of SCFA, phenol, and *p*-cresol in cecal digesta of rats; values are mean ± SEM, n = 8 (except SCFA of the PF group n = 7) (each sample analysed in duplicate). Means in a row with different superscripts indicate significant differences (P < 0.05).

Gut microbial metabolites	Control	WS diet	PF diet
Cecal SCFA pool[a] *(μmol)*			
Acetic acid	92.44^{b} ± 6.98	138.12^{a} ± 9.07	122.71^{a} ± 12.63
Propionic acid	30.93^{b} ± 2.59	49.21^{a} ± 3.60	46.93^{a} ± 5.29
Butyric acid	26.95^{a,b} ± 3.55	21.40^{b} ± 2.56	33.73^{a} ± 6.29
Valeric acid	2.99^{a} ± 0.36	2.29^{a} ± 0.31	1.40^{b} ± 0.43
Caproic acid	0.63^{a} ± 0.25	0	0.07^{b} ± 0.07
iso-Butyric acid	3.12^{a} ± 0.51	1.20^{b} ± 0.22	1.56^{b} ± 0.21
iso-Valeric acid	2.89^{a} ± 0.50	1.03^{b} ± 0.20	2.19^{a} ± 0.36
Total SCFA	159.96^{b} ± 13.10	213.25^{a} ± 14.40	208.59^{a} ± 23.32
SCFA concentration (μmol/g cecal digesta)			
Acetic acid	51.23^{a} ± 3.39	48.18^{a} ± 2.11	27.57^{b} ± 2.65
Propionic acid	16.87^{a} ± 0.54	17.12^{a} ± 0.84	10.31^{b} ± 0.79
Butyric acid	14.32^{a} ± 1.18	7.35^{b} ± 0.68	7.60^{b} ± 1.35
Valeric acid	1.59^{a} ± 0.08	0.79^{b} ± 0.08	0.34^{c} ± 0.11
Caproic acid	0.35^{a} ± 0.15	0	0.01^{b} ± 0.01
iso-Butyric acid	1.61^{a} ± 0.13	0.41^{b} ± 0.07	0.34^{b} ± 0.03
iso-Valeric acid	1.48^{a} ± 0.14	0.35^{b} ± 0.06	0.47^{b} ± 0.05
Total SCFA	87.45^{a} ± 3.86	74.20^{b} ± 2.74	46.65^{c} ± 4.53
Cecal phenol pool[a] (μg)	5.77^{a} ± 1.61	1.00^{b} ± 0.07	2.07^{b} ± 0.15
Phenol concentration (μg/g cecal digesta)	2.91^{a} ± 0.70	0.36^{b} ± 0.03	0.49^{b} ± 0.02
Cecal *p*-cresol pool[a] (μg)	25.18^{a} ± 6.18	1.32^{b} ± 0.12	19.34^{a} ± 5.14
p-cresol concentration (μg/g ceccal digesta)	11.96^{a} ± 2.20	0.47^{b} ± 0.05	4.45^{b} ± 0.97

[a] Cecal SCFA, phenol, and *p*-cresol pool mean total SCFA, phenol, and *p*-cresol amounts in the cecal digesta by taking the weight of the cecal digesta into account.

observed in the significant decrease in the level and concentration of cecal *p*-cresols of rats fed with WS and PF diets when compared to the control group. The WS diet resulted in approximately 95% decreases in level and concentration of cecal *p*-cresols, however the PF diet only resulted in a 63% decrease in concentration but the level (which takes the weight of the cecal digesta into account) was not statistically significant.

3.4. Bacterial enumeration

The selected bacterial populations in rat cecal digesta resulting from different dietary supplementation are shown in Table 5.

Table 5
Effect of WS and PF diets on selected bacterial population in cecal digesta of rats from which DNA was extracted in duplicate; values are mean ± SEM, n = 8. PCR amplification was carried out in triplicate from each of these DNA samples. Means in a row with different superscripts indicate significant differences (P < 0.05).

Bacteria	Log_{10} bacteria/total cecal digesta (g)		
	Control	WS diet	PF diet
Bifidobacterium	6.24^{a} ± 0.20	4.83^{b} ± 0.31	5.41^{b} ± 0.13
Lactobacillus	7.98^{a} ± 0.11	7.37^{b} ± 0.11	7.62^{a,b} ± 0.12
Faecalibacterium prausnitzii	4.87^{b} ± 0.11	4.99^{ab} ± 0.14	5.32^{a} ± 0.11
Clostridium coccoides	8.21^{a} ± 0.07	7.80^{b} ± 0.08	8.05^{ab} ± 0.13
Bacteroidetes	7.40^{a} ± 0.07	7.27^{a} ± 0.08	7.48^{a} ± 0.10
Bacteroides-Prevotella	7.43^{a} ± 0.08	7.32^{a} ± 0.10	7.45^{a} ± 0.12
Firmicutes	8.40^{a} ± 0.09	7.93^{b} ± 0.08	8.50^{a} ± 0.07
Enterococcus	6.04^{a} ± 0.09	5.59^{b} ± 0.08	5.96^{a} ± 0.10
Escherichia coli	5.24^{b} ± 0.19	5.10^{b} ± 0.16	6.38^{a} ± 0.14
Total Bacteria	8.35^{a} ± 0.08	7.93^{b} ± 0.08	8.44^{a} ± 0.06
Ratio of *Firmicutes: Bacteroidetes*	1.13^{a} ± 0.006	1.09^{b} ± 0.005	1.14^{a} ± 0.100

Relative to the control diet group, the numbers of key butyric acid producer *F. prausnitzii* in the cecal digesta of rats fed with PF diet significantly increased, while a decrease in numbers of *Enterococcus* in the cecal digesta of rats fed with WS diet was observed. Additionally, significantly higher abundance, approximately 10-fold, of *E. coli* in the rat cecal digesta fed with PF diet was observed in comparison to that of the control and WS diets. The *Firmicutes* to *Bacteroidetes* ratio was calculated for each diet group, and the lowest of these ratios was found in the rat cecal digesta fed with WS diet. However, a decrease in the number of *Bifidobacterium* and *Lactobacillus* was detected in the rats in two treatment groups when compared to the control. The lowest abundance of *C. coccoides*, *Firmicutes*, and total bacteria were noticed in rats fed with WS diet.

3.5. Characterisation of the PF

After selective extractions, it was found that the alginate and fucoidan accounted for 23.8% ± 1.7 and 46.1% ± 1.7 (dry weight) of the PF, respectively. Approximately 30% of the remaining components could be accounted for by ash (~20%) and small amounts of other components (~10%) such as protein, phlorotannin, fat, etc. (data not shown). The MWs of alginate and fucoidan in the fraction were then estimated by HPGPC, relative to the dextran standards. Both alginate and fucoidan showed a single peak in the chromatograms (data not shown), indicating their homogeneity. The peak MW of alginate and fucoidan was estimated to be 237.03 and 339.78 kDa, respectively. The sulphur content of fucoidan fraction (7.65% ± 0.16) was analysed by ICP-OES indicated that the fucoidan fraction contained 24.62% ± 0.52 sodium sulphate.

The identification of alginate and fucoidan was further confirmed by spectroscopic methods (FT-IR and NMR), and their corresponding spectra are shown in Figs. 1 and 2.

Alginate The FT-IR spectrum of alginate contained intense absorption bands at 1618 and 1420 cm^{-1} (carboxyl group; C=O stretching), which is characteristic of alginates (Imbs, Ermakova, Malyarenko, Isakov, & Zvyagintseva, 2016). An additional broad band at 3359 cm^{-1} was assigned to hydrogen bonded O—H stretching vibrations, and the signal at 2933 cm^{-1} was attributed to C—H stretching. More signals at 891 and 819 cm^{-1} were assigned to the α-L-gulopyranuronic asymmetric ring vibration and to the mannuronic acid residues, respectively (Fenoradosoa et al., 2010). In the ^{13}C NMR spectrum of the alginate fraction, there are three regions consisting of C2-C5 (60–90 ppm), anomeric (C1, 90–110 ppm), and carboxyl (C6, 172–180 ppm) carbon signals. The major chemical shifts were detected at 178.04, 104.75, 103.95, 83.49, 80.60, 78.54, 74.09, 73.52, 72.69, 71.82, 67.47 ppm which could refer to G6 M6, G1, M1, G4, M4, M5, M3, M2, G3, G5, and G2, respectively (Salomonsen, Jensen, Larsen, Steuernagel, & Engelsen, 2009). For ^{1}H NMR, the characteristic anomeric proton signal of polymannuronic acid appeared at 4.71 ppm. The signals at 4.44 and 5.02 ppm were indicative for the presence of some guluronic acid residues (Chandía, Matsuhiro, Mejías, & Moenne, 2004).

Fucoidan The FT-IR spectrum of fucoidan contained an intense absorption band at 1261 cm^{-1} attributed to asymmetric O=S=O stretching vibration of sulphate esters with some contribution of COH, CC, and CO vibrations, which is a typical characteristic of sulphated polysaccharides (Synytsya et al., 2010). An additional sulphate absorption band at 826 and 850 cm^{-1} were attributed to C—O—S, secondary equatorial sulphate at C-2 or C-3 and axial sulphate at C-4, respectively (Kim et al., 2010). The ^{13}C NMR spectrum of fucoidan was complex. Results showed that C6 carbon signals of neutral sugar units were found at 18.62 ppm (CH_3 of α-L-fucopyranose units), and corresponding methyl carbon signals at

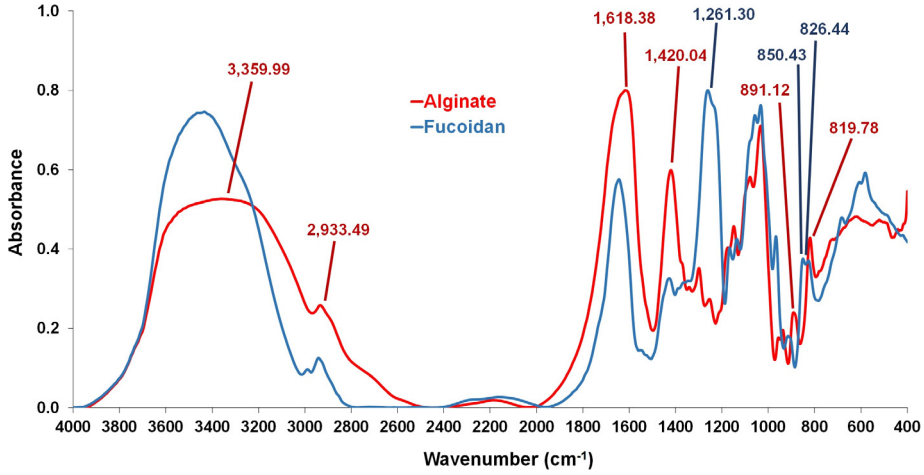

Fig. 1. FT-IR spectra of alginate and fucoidan separated from the PF of seaweed *E. radiata*.

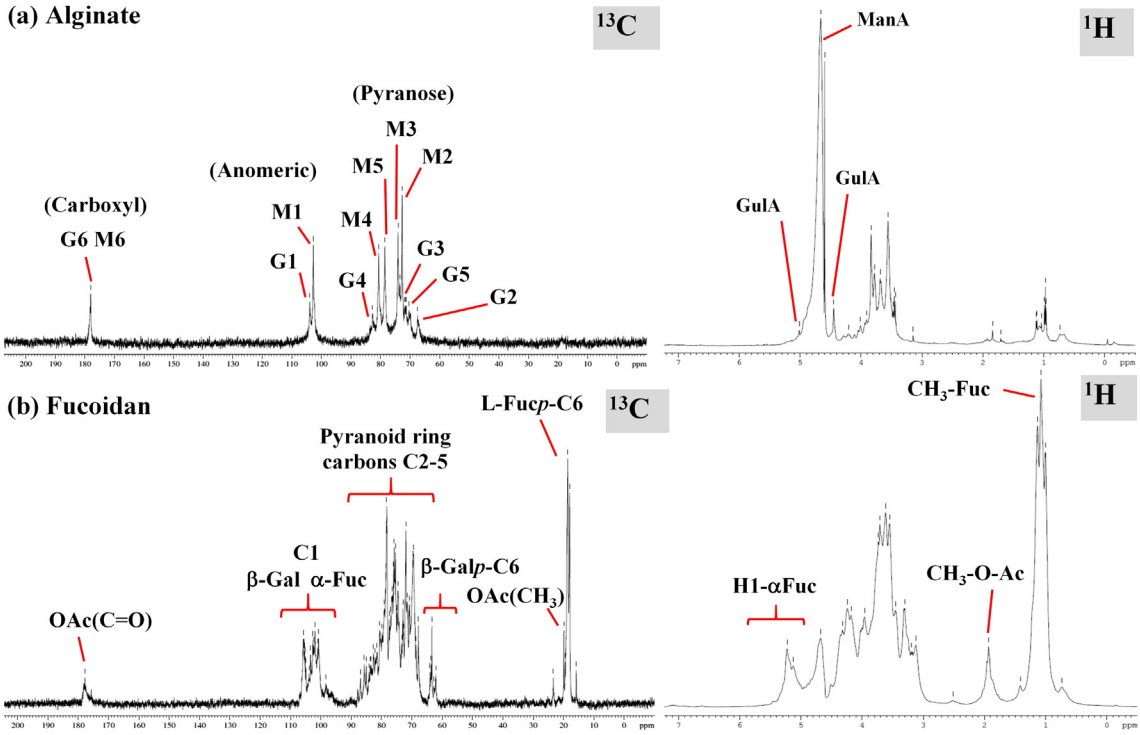

Fig. 2. ^{13}C and ^{1}H NMR spectra of (a) Alginate and (b) Fucoidan separated from the PF of seaweed *E. radiata*.

19.89 ppm were assigned to *O*-acetyl groups. The bands at 62.06 and 64.04 ppm were assigned to CH_2OH of β-D-galactopyranose units and CH_2OR of β-D-galactopyranose substituted at O6, respectively. The region 65–83 ppm contains complex signals of pyranoid ring carbons C2-5. Several resonance signals of anomeric (C1) carbons were found around 95–105 ppm, and a carbonyl carbon signal was observed at 177.79 ppm (Ermakova et al., 2011; Synytsya et al., 2010). The signals of ^{1}H NMR spectrum in the last region at 1.00 and 1.13 ppm were assigned to C6 methyl protons of L-fucopyranose, and a signal at around 2 ppm arose from CH_3 protons of *O*-acetyl groups. Signals in the α-anomeric were found at 5.0–5.6 ppm assigned mainly to H1 of α-L-fucopyranose residues and CH protons of *O*-substituted carbons (Bilan et al., 2004; Synytsya et al., 2010).

4. Discussion

In this study, brown seaweed *E. radiata* and its derived polysaccharides were examined as potential sources of prebiotics using a rat model. Rats were fed with diets supplemented with 5% of WS or PF (which we show here to be rich in fucoidan and alginate) for 1 wk and effects compared with those of a standard (control) diet. We show that the WS and/or the PF are able to undergo fermentation by gut microbes *in vivo*, resulting in the production of beneficial SCFA, stimulate an increase in digesta bulk, and alter some populations of microbes which can positively influence gut health.

The increases in mass or length of gut tissue and digesta of the large bowel in rats fed with PF diet are typical effects of dietary

fibre on the gut (Eastwood, 1992). These effects could be explained by the high levels of dietary fibre and NNSP present in the PF. The PF diet had significant impacts on the cecum and colon of rats. In comparison to other studies, the 2-fold increase in total cecum weight of rats fed with this PF diet compared with the control group was higher than the increase in cecum weight of rats fed with 6% (w/w) of the prebiotics fructo-oligosaccharide (1.7-fold) and oligofructose (1.9-fold), but lower than xylooligosaccharides (2.4-fold), when fed for a period for 2 wks (Campbell, Fahey, & Wolf, 1997). In addition, relative to the control group, the increase in colon weight (1.4-fold) and length (1.1-fold) of rats fed with this PF diet was comparable to the increase in colon weight and length (1.2-fold) of obese leptin-deficient mice fed with 10% (w/w) of the prebiotic oligofructose-supplemented diet for 5 wks (Everard et al., 2011). The increase in fecal moisture content in rats fed with PF diet was likely a sign of the elevated water holding capacity of the fibre and might be related to the increase in the growth of colonic mucosa (Sircar, Johnson, & Lichtenberger, 1983).

Significant improvements of SCFA production in rats fed with WS and PF diets compared to the control group were most likely a result of the fermentable fibres in the WS and PF. de Jesus Raposo et al. (2016) indicated that the fermentation of seaweed polysaccharides by beneficial bacteria have been shown to generate SCFA, which can have different physiological impacts within the gut. Butyric acid is the primary energy source of cells lining in the colon, and helps maintain colonic tissue integrity through stimulation of apoptosis in cells with high levels of DNA damage (Canani et al., 2011). Acetic acid can inhibit the growth of enteropathogenic bacteria (Fukuda et al., 2011), and propionic acid produced in the gut may influence hepatic cholesterol synthesis (Raman, Ambalam, & Doble, 2016). In our study, the most abundant SCFA in rat cecal digesta were acetic acid followed by propionic acid and butyric acid, with low concentrations of the branched chain fatty acids. This result was similar to the SCFA profiles obtained from previous *in vitro* fermentations of the WS and PF (Charoensiddhi et al., 2016, 2017).

Putrefactive microbial products derived from protein fermentation include phenols and *p*-cresol. The accumulation of these compounds in the gut are linked to loss of gut integrity and function, and increased risk of bowel diseases (Windey, De Preter, & Verbeke, 2012). The rats fed with WS and PF diets tested in this study produced significantly lower levels and concentrations of phenol and *p*-cresol in cecal digesta than the control. This might be associated with their polysaccharide content as it has been reported that the fermentation of alginate by gut microbes could suppress the formation of putative risk markers for colon cancer (Kuda, Yano, Matsuda, & Nishizawa, 2005). The WS diet was more effective in reducing phenol and *p*-cresol contents in rat cecal digesta compared to the PF diet group. This result may be related to the 3-fold greater phlorotannin content in the WS. Other studies suggest dietary polyphenols can have such an effect. For instance, a decrease in phenol and *p*-cresol in feces were found in humans after consuming a grape seed extract supplemented diet for 2 wks (Yamakoshi et al., 2001). Similar results with the decrease in phenol and *p*-cresol concentrations in pig feces was observed after 2 wks feeding with a tea phenol-enriched diet (Hara et al., 1995). Further studies to understand the roles of seaweed phenolic compounds on protein fermentation are still required. However, a decrease in pH of the feces to acidic resulting from bacteria fermentation after phenolic intervention may decrease the concentration of putrefactive compounds as neutral or slightly alkaline are more optimal conditions for microbial proteases (Macfarlane, Allison, & Gibson, 1988). In addition, phenolic compounds are reported to inhibit the growth of proteolytic bacteria and reduce the expression of genes involved in the production of proteases (Mosele, Macià, & Motilva, 2015).

In this study, we used Q-PCR to monitor changes in populations of selected colonic bacteria populations in response to diets supplemented with WS and PF. An increase in the key butyric acid producer *F. prausnitzii* was observed in the cecal digesta of rats fed with the PF diet compared to the control, concurrent with an increase in butyric acid production. This bacterium is of particular interest because in addition to its ability to generate butyrate it has been identified as having anti-inflammatory effects which may help protect against inflammatory bowel disease (Miquel et al., 2013; Sokol et al., 2008). The use of an enzyme-assisted extraction process has resulted in modification and/or breakdown of some high MW components, including polysaccharides, in the PF, which may improve the activity or bioavailability of lower MW components that promote the growth and activity of *F. prausnitzii*. A decrease in the cecal numbers of the potentially pathogenic *Enterococcus* was observed when rats were fed the WS diet. Other *E. radiata* compounds present in WS but not the PF, particularly polyphenols, may have contributed to this effect, as polyphenols have previously been shown capable of regulating of pathogenic microbial activity (Eom, Kim, & Kim, 2012). Feeding with WS resulted in a reduced ratio of the *Firmicutes*/*Bacteroidetes*. This may be beneficial to human health as it is associated with a decreased risk of obesity or excessive body weight (Ley, Turnbaugh, Klein, & Gordon, 2006; Ley et al., 2005). An increase in the number of *E. coli* was observed in the cecal digesta of rats fed with the PF diet. Although *E. coli* are commonly known for their pathogenic potential, they can also have some benefits as a consequence of non-pathogenic strains outcompeting the pathogenic forms, as is thought to occur for the probiotic *E. coli* Nissle 1917 (Gerritsen, Smidt, Rijkers, & Vos, 2011). The abundance of some beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, traditional markers of prebiosis, were not increased by diets in our study. Despite these bacteria not changing, a significant increase in the beneficial activity of gut bacteria is nevertheless suggested by the increase in cecal SCFA. It is quite likely that the populations or activities of other beneficial bacteria were also increased, but these were not analysed here and would need to be examined in future studies.

A few studies have previously demonstrated gut health benefits (prebiotics effects) of alginates and fucoidans. Kuda et al. (2005) reported that rats fed with 2% (w/w) of a low MW (49 kDa) alginate-supplemented diet could increase cecum weight and total SCFA production, and decrease indole and *p*-cresol levels, when compared with the control group. In another rat study, a 2.5% (w/w) alginate oligosaccharide-supplemented diet stimulated the growth of fecal bifidobacteria by 13-fold and 4.7-fold when compared with a control diet and a diet supplemented with 5% fructo-oligosaccharides, respectively. In addition, the alginate supplemented diet resulted in a significant increase in *Lactobacillus* and decreases in *Enterobacteriaceae* and *Enterococcus* compared with the control group (Wang, Han, Hu, Li, & Yu, 2006). Pigs fed with a 238 ppm of fucoidan-supplemented diet significantly increased their colonic *Lactobacillus* populations and total SCFA production (Lynch, Sweeney, Callan, O'Sullivan, & O'Doherty, 2010). Shang et al. (2016) also demonstrated that mice fed with fucoidan (100 mg/kg/day) increased the abundance of *Lactobacillus* and *Ruminococcaceae*, and decreased the number of pathogenic *Peptococcus* in cecum. In our study, when rats were fed with the PF diet we did not detect an increase in the abundance of *Bifidobacterium* or *Lactobacillus*, nor did we find a decrease in the potentially pathogenic bacteria we examined.

Alginate and fucoidan are major polysaccharides found in brown seaweeds (Holdt & Kraan, 2011) and were expected to be present in *E. radiata*. To confirm their presence, alginate and fucoidan were each selectively isolated from the PF and characterised. The PF contained primarily fucoidan (46%) and alginate (24%).

The FT-IR spectrum was in complete agreement with the ^{13}C and ^{1}H spectra, indicating that mannuronic acid was predominant in the alginate. This result corresponded to the NMR spectra reported by Salomonsen et al. (2009) for commercial sodium alginate powders containing 65% mannuronate. Moreover, low viscosity was observed for this mannuronic acid-rich alginate, and the addition of calcium did not significantly promote gelation (data not shown). These observations further support the assertion that the alginate was dominated by mannuronic acid, as the polyguluronic acid (G) blocks can bind calcium ions between two chains more effectively than the polymannuronic acid (M) blocks, resulting in higher viscosity and stronger gel forming capabilities (Sari-Chmayssem et al., 2016). For fucoidan, the carbon signals of NMR were split into several peaks, which confirm the presence of several structural patterns of sugar units, depending on the substitution. It may indicate that this fucoidan is galactofucan, sulphated and acetylated at different positions of the galactose and fucose residues. It was also worth noting that the sulphate group in this fucoidan (24.6%) was relatively higher than that of other brown seaweed species, such as *Ascophyllum nodosum* (22.3%), *Fucus vesiculosus* (19.0%), and *Saccharina longicruris* (14.2%) analysed using the same technique (Rioux et al., 2007). The bioactivity of many fucoidans, such as anticoagulant activity, is enhanced with increased sulphatation (Li, Lu, Wei, & Zhao, 2008), so the role of highly sulphated fucans on the prebiotic properties of these polysaccharides should be investigated further.

Further studies covering a different range of seaweed-derived polysaccharide components and structures are suggested in order to more fully understand which specific components in seaweeds are responsible for gut health effects, and whether polysaccharide mixtures could be tailored to optimise these health benefits. Also, the characterisation and development of low MW seaweed polysaccharides may facilitate dietary formulations which improve fermentability by gut microbes (Ramnani et al., 2012).

5. Conclusion

Our study has shown for the first time that the brown seaweed *E. radiata* and its polysaccharide fraction can improve different aspects of gut health *in vivo*. Relative to the control group, rats fed the WS diet produced significantly higher levels of SCFA and lower levels of phenol, *p*-cresol, and potentially pathogenic *Enterococcus*, whereas rats fed with the PF diet significantly increased their cecum and colon weights, cecal SCFA production, and the abundance of *F. prausnitzii*, a primary butyrate producer with anti-inflammatory actions. Sulphated fucan and mannuronic acid-rich alginate with approximate MW of 339.8 and 237.0 kDa respectively, are the predominant components of PF and hence most likely to be responsible for any benefits which we have described. In addition, the higher phlorotannin content in the WS compared to the PF might play a role in the inhibition of phenol and *p*-cresol production and pathogenic bacteria growth. These findings suggest that *E. radiata* and its polysaccharides have the potential to be used as a dietary supplement with gut health benefits in humans.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.08.040.

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