Journal of Functional Foods 96 (2022) 105204

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Gut microbiome modulation and gastrointestinal digestibility *in vitro* of polysaccharide-enriched extracts and seaweeds from *Ulva rigida* and *Gracilaria fisheri*

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ARTICLE INFO

Keywords:
Dietary fibers
Digestibility
Gut microbiome
Macroalgae
Prebiotic
Short-chain fatty acids

ABSTRACT

This study explored the gut health potential of the green seaweed *Ulva rigida* (SW-U) and the red seaweed *Gracilaria fisheri* (SW-G), as well as polysaccharide-enriched extracts (PF-U and PF-G, respectively). The polysaccharide-enriched extracts were not digestible by small intestinal enzymes, but the morphology of samples was changed. After 24 h *in vitro* fermentation, seaweeds and polysaccharide-enriched extracts significantly increased ($p < 0.05$) production of total short-chain fatty acids (SCFAs) (29.4–35.4 μmoL/mL) compared to the control (17.9 μmoL/mL). The *G. fisheri* showed more potential for improving gut health than *U. rigida*. Particularly, PF-G induced butyric acid production comparable to that of inulin control (5.4 and 6.9 μmoL/mL) and the highest production when compared with other substrates (1.8–3.3 μmoL/mL). While SW-G stimulated the growth of beneficial bacteria, including *Roseburia* and *Faecalibacterium*. These findings further demonstrate that seaweeds and their derived polysaccharides have the potential to be used as dietary supplements with gut health benefits.

1. Introduction

Marine macroalgae or seaweeds are diverse groups of mostly photosynthetic organisms, which are considered important sources of bioactive compounds with a range of biological activities (Gupta and Abu-Ghannam, 2011). They are rich in both primary and secondary metabolites such as carbohydrates, proteins, polyunsaturated fatty acids (PUFAs), polyphenols, and pigments, including several minerals (Holdt and Kraan, 2011). Seaweed bioactive compounds possess various biological functions demonstrated both *in vitro* and *in vivo* studies (Biris-Dorhoi et al., 2020). For instance, sulfated polysaccharides showed anticoagulant and antithrombotic properties, while polyphenols exhibited the antihyperglycaemic, antihyperlipidaemic, anti-inflammatory, and antioxidant effects. Additionally, alginate and porphyran were also shown to decrease the blood sugar levels, and fucoidan could modulate the immunity. The cultivation of seaweeds has been growing rapidly, and most of these seaweeds are used for direct consumption, raw materials for food production, hydrocolloids, and fertilizers. The wild collection remained at 1.1 million tonnes, while the cultivation increased to 34.7 million tonnes, accounting for 97 percent of world seaweed production in 2019 (Cai et al., 2021). Although seaweed bioactive compounds are attractive for commercialization in different functional food and nutraceutical products, the use of seaweed for this purpose is still not extensive. The consumption and utilization of seaweed are more associated with the applications of unique seaweed-based hydrocolloids valued approximately USD 1.74 billion in 2019

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https://doi.org/10.1016/j.jff.2022.105204
Received 28 December 2021; Received in revised form 10 July 2022; Accepted 25 July 2022
Available online 30 July 2022

(Cai et al., 2021).

The gut microbiota plays an important role in human health, particularly protecting the intestinal epithelial barrier, regulating the immune system, preventing adhesion of pathogens to intestinal epithelial cells, and producing beneficial metabolites such as short-chain fatty acids (SCFAs) (Zhang et al., 2015). Recently, there is increasing evidence that diet is a key factor influencing the composition of the gut microbiota, and the intake of fibers, especially prebiotic inulin and fructo-oligosaccharide, can manage a number of gastrointestinal disorders (Gill et al., 2021). Interest in prebiotics as functional foods has increased due to their recognized health benefits. The global prebiotics market was valued at USD 8.95 billion in 2020 and is projected to grow at a CAGR of 7 % during the forecast period till 2030 due to increasing consumer awareness of gut health issues (Quince Market Insights, 2021). O'Sullivan et al. (2010) and Charoensiddhi et al. (2020) suggested that most seaweed polysaccharides such as alginates, fucoidans, laminarins, ulvans, agars, and carrageenans may be regarded as dietary fibre, as they resist the digestion by enzymes present in the human gastrointestinal tract, selectively stimulate the growth of beneficial gut bacteria, inhibit pathogen adhesion and evasion, and modulate intestinal metabolism including fermentation. The fermentation of seaweed components by beneficial bacteria has also been shown to generate beneficial metabolites such as SCFAs, particularly butyrate, acetate, and propionate (de Jesus Raposo et al., 2016). The beneficial effects of SCFAs exert many positive effects on host health and disease, including obesity, chronic respiratory, and inflammatory bowel, as well as modulate the immunity, glucose homeostasis, lipid metabolism, and appetite regulation (Bultman, 2016; Dwivedi et al., 2016; Koh et al., 2016; Morrison and Preston, 2016). Also, the beneficial changes in gut microbes in response to prebiotics is well documented, and this has been demonstrated for seaweed-derived prebiotics in several studies. For instance, Charoensiddhi et al. (2016; 2017) demonstrated that extracts from the brown seaweed *Ecklonia radiata* stimulated the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacillus* which are the most commonly recognized bacterial markers of prebiosis (Kleerebezem and Vaughan, 2009; Bird et al., 2010). Kuda et al. (2015) demonstrated that sodium alginate and laminaran from brown seaweeds inhibited the adhesion and invasion of pathogens (*Salmonella Typhimurium*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*) in human enterocyte-like HT-29-Luc cells.

Red and green seaweeds generally contain relatively high levels of polysaccharides that could be developed as prebiotics, and this appears to be the case for the most commonly exploited Thai red and green seaweeds *Gracilaria* and *Ulva*. Benjama and Masniyom (2011, 2012) reported that red seaweeds *Gracilaria fisheri* and *G. tenuistipitata* from the Pattani Bay in Southern Thailand contained total dietary fibre (TDF) 57.5–64.0 % dry weight (DW), while green seaweeds *Ulva pertusa* and *U. intestinalis* contained TDF 51.3–62.2 % DW. Although the commercial products and patent activities on seaweed-derived bioactive compounds have increased recently, only a few published studies have reported on the gut health potential of polysaccharides from green and red seaweeds (Bobin-Dubigeon et al., 1997; Andrieux et al., 1998; Hu et al., 2006; Zhang et al., 2020), and none of them have examined the *Ulva rigida* and *Gracilaria fisheri*. Therefore, this study aims to investigate the gut health potential of both seaweeds including their polysaccharide-enriched extracts through investigation of their *in vitro* digestibility and their ability to be fermented *in vitro* by human fecal gut microbes. Their influences on gut microbial populations and their activity, especially the ability to stimulate production of beneficial gut fermentation products such as SCFAs were examined.

2. Materials and methods

2.1. Materials

Green seaweed (*Ulva rigida*) and red seaweed (*Gracilaria fisheri*) collected from a seaweed farm in the South of Thailand during May to June were supported by Phetchaburi Coastal Fisheries Research and Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives. After harvesting, they were washed in tap water to remove any visible surface contaminants and placed on mesh racks to dry. Then, they were dried in an oven at 60 °C for 8 h to obtain a moisture content of approximately 10 %, finely ground using a hammer mill and sieved through 0.2 mm mesh. All seaweeds were collected and prepared at the one time to obtain the consistent samples for this study. The ground sample powder is stored at room temperature in a desiccator prior to extraction and fermentation. All chemicals used were of analytical or chromatography grade from Merck or Sigma (if specify).

2.2. Polysaccharide extraction

For preparation of polysaccharide-enriched extracts from *U. rigida*, proteins in seaweed samples were removed using the modified method of Vilg and Undeland (2017) prior to polysaccharide extraction. Briefly, dried and ground seaweed was suspended in the distilled water with a 1:20 (w/v) ratio based on DW of seaweed for 1 h at room temperature. Then, pH was adjusted to 12 with 1 M NaOH, and the seaweed slurry was further stirred for 2 h at room temperature. Then, polysaccharide was extracted using the modified method described by Yaich et al. (2013). The residual from protein extraction was suspended in the distilled water with a 1:10 (w/v) ratio at 90 °C for 3 h. The supernatant was then separated by centrifugation at 8000*g* for 20 min. Ethanol (>95 %) was then added to the supernatant in order to obtain the final concentration of 67 % (v/v) to precipitate the polysaccharide. Next, the obtained polysaccharide extract (PF-U) was freeze dried.

Extraction of polysaccharide from *G. fisheri* (PF-G) was carried out in the same manner as mentioned above, with the exception of the seaweed:water ratio (1:27 w/v). After extraction, HCl was added to seaweed slurry to achieve the final concentration of 0.02 M, and the mixture was stirred at 90 °C for further 1 h (modified method from Kazłowski et al., 2015). The seaweed slurry was centrifuged. The pH of supernatant was adjusted to 7, prior to polysaccharide precipitation in ethanol. The polysaccharide was dried by freeze-dryer (Gamma 2–16 LSC, Martin Christ GmbH, Osterode am Harz, Germany) for further study.

2.3. Characterization of seaweeds and their polysaccharide-enriched extracts

2.3.1. Proximate and compositions of U. rigida and G. fisheri seaweeds

Moisture, protein (Kjeldahl- N × 6.25), total fat (Mojonnier), ash (ignition at 550 °C), carbohydrate (Calculation), and dietary fiber contents based on DW of the prepared seaweed powders were analyzed by standard methods of Central Laboratory (Thailand) Co., ltd. according to the Association of Official Analytical Collaboration (AOAC) International guideline.

Sulfate content was determined using a turbidimetric method (Peasura et al., 2015). A dried sample (2 mg) was digested with 0.2 mL of 1 N HCl at 95 °C for 5 h. After the samples were cooled at room temperature and centrifuged 2000 × g, 4 °C, 5 min, 190 μL of 3 % trichloroacetic acid was added to 10 μL of sample. Barium chloride–gelatin reagent (50 μL; prepared as 2 g gelatin and 2 g barium chloride in 400 mL of water) was added, and the mixture was left for 20 min at room temperature. The absorbance was read at 360 nm by microplate reader, and the sulfate content was calculated based on the standard curve of sodium sulphate solutions (0.5–3 mg/mL dissolved in 1 N HCl).

2.3.2. Chemical compositions of polysaccharide-enriched extracts

The total carbohydrate was determined by phenol–sulfuric acid method using rhamnose (for PF-U) and galactose (for PF-G) as a standard (Dubois et al., 1956; Kazir et al., 2019). Briefly, 150 μL of sulfuric acid (95–97 %) were added rapidly to 50 μL of sample or standard, along with 30 μL of 5 % phenol (≥99.5 %). After 30 min of incubation at room

temperature, the absorbance at 490 nm was measured by spectrophotometer.

The uronic acid was quantified by sulphuric acid hydrolysate method (uronic acid carbazole reaction) using D-glucuronic acid as a standard (Bitter and Muir, 1962; Yaich et al., 2014). Briefly, 200 μL of 25 mM sodium tetraborate in sulfuric acid were added to 50 μL of sample or standard, then heated for 10 min at 100 °C. After cooling at room temperature for 15 min, 50 μL of 0.125 % carbazole (≥95 %) in absolute ethanol were added, then heating at 100 °C for 10 min and cooling at room temperature, the absorbance at 550 nm was measured by microplate reader.

The total protein was determined based on the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951). Briefly, the reaction mixture consisting of 50 μL of sample and 150 μL of freshly made reagent A (1 part 4 % $CuSO_4 \cdot 5H_2O$ into 100 parts of a mixture of 2.0 % Na_2CO_3, 0.40 % NaOH, 0.16 % Na-tartrate, and 1 % SDS) was incubated for 30 min at room temperature. Then, 15 μL of freshly made reagent B (1 part of Folin-Ciocalteu phenol reagent into 1 part of Milli-Q water) was added and incubated for 45 min in darkness at room temperature. The absorbance was measured at 750 nm by microplate reader.

Sulfate content was carried out in the same manner as described in Section 2.3.1.

Monosaccharide compositions of samples were analyzed using a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), following a method of Methacanon et al. (2014). Polysaccharide samples (50 mg) were hydrolyzed with 5 mL of 90 % (v/v) formic acid and incubated at 100 °C for 2 h. After removal of the formic acid, 0.125 M of sulfuric acid (12.5 mL) was added to samples and incubated at 100 °C for 10 h. The hydrolysate was neutralized with barium carbonate and centrifuged at 10,000*g*, 4 °C for 30 min. Then, the pallets were washed with distillated water 1 mL for three times. All supernatants from washing were collected and distilled in a parallel evaporator. Distillated water (1 mL) was added to dissolve the pallet from evaporation, and centrifuged at 10,000*g*, 4 °C for 30 min. Then samples were diluted to the final concentration of 1 mg/mL before injection.

Molecular weight (MW) of samples were analyzed using 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 1,000–20,000,000) with a guard column and a refractive index detector. Polysaccharide fractions 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).

2.3.3. FT-IR spectroscopy

The infrared spectra of seaweeds and polysaccharide-enriched extracts were recorded using FT-IR Spectrometer (Bruker Tensor 27, Bremen, Germany) equipped with a MIR detector. The dried samples were ground together with potassium bromide (KBr), and the mixture of each sample was pressed as a pellet under high pressure. A region from 400 to 4000 cm^{-1} was used for scanning at 4 cm^{-1} resolution over 32 scans.

2.4. In vitro gastrointestinal digestion of polysaccharide extracts

The method of simulated gastrointestinal digestion was performed according to Minekus et al. (2014) with slight modification. The initial sample solutions (6 mL) containing 2 % (w/v) polysaccharide extract (distilled water used as a control) were mixed with 6 mL of Simulated Salivary Fluid (SSF) working solution containing 4.2 mL of pre-heated SSF stock solution, 0.6 mL of α-amylase solution 1,500 U/mL (α-amylase from porcine pancreas Type VI-B, ≥ 5 units/mg solid, Sigma), 30 μL of 0.3 M $CaCl_2$, and 1.2 mL of distilled water. After mixing, the pH of the sample-SSF mixers were adjusted to 7, and then they were incubated at 37 °C with shaking for 5 min. The samples from the oral phase (10 mL) were mixed with 10 mL of Simulated Gastric Fluid (SGF) working solution containing 7.5 mL of pre-heated SGF stock solution, 1.6 mL of pepsin solution 25,000 U/mL (pepsin from porcine gastric mucosa ≥ 250 units/mg solid, Sigma) and 5 μL of 0.3 M $CaCl_2$ and 0.9 mL of distilled water. The sample-SGF mixers were adjusted to pH 3 and then incubated at 37 °C with shaking for 2 h. The samples from the gastric phase (18 mL) were mixed with 18 mL of Simulated Intestinal Fluid (SIF) working solution containing 9.9 mL of pre-heated SIF stock solution, 4.5 mL of pancreatin solution 800 U/mL (Pancreatin from porcine pancreas 8 × USP based on trypsin activity, Sigma), 2.25 mL of bile solution (160 mM fresh bile, Sigma) and 36 μL of 0.3 M $CaCl_2$ and 1.4 mL of distilled water. The sample-SIF mixers were adjusted to pH 7 and then incubated at 37 °C with shaking for 2 h. The oral, gastric, and intestinal digestive samples were collected. The reaction was terminated by immersing tubes into liquid nitrogen and stored at −80 °C immediately prior to freeze dried for further analyses, i.e., determination of reducing sugar content, particle size, and morphology.

2.4.1. Reducing sugar content

The total reducing sugar of undigested and digested samples was measured by Dinitro Salicylic Acid (DNS) method using rhamnose (for PF-U) and galactose (for PF-G) as a standard (Miller, 1959; Garriga et al., 2017). To determine the total reducing sugar, the sample solutions (each phase of digestion) were centrifuged at 6500*g* for 5 min to obtain the supernatant. Briefly, 100 μL of the supernatant was mixed with 100 μL of DNS reagent. After mixing, the tube was incubated in a water bath at 95 °C for 5 min, and then cooled to room temperature. Then 200 μL of the solution was diluted with 800 μL of DI water to obtain 1 mL of final solution before measuring the absorbance at 540 nm by microplate reader.

2.4.2. Particle size distribution

The average diameter and particle size distribution of undigested and digested samples in water were determined using a Zetasizer Nano-ZS with the dynamic light scattering (DLS) (Zen 3600, Malvern Instruments ltd., UK) at 25 °C. The particle size distribution was reported as the polydispersity index (PDI).

2.4.3. Morphology

The morphology of undigested and digested samples was analyzed by Field Emission Scanning Electron Microscopy, FE-SEM (SU-8020, Hitachi, Japan). The sample powders were taped and placed on a 15 mm diameter stub which was attached with a carbon tap, then coated with platinum using sputtering equipment (Quorum Q150R ES, Quorum Technologies ltd., UK) to increase the electrical conductivity of samples before analysis.

2.5. In vitro human gut model

Fresh fecal samples were provided by three individual healthy human volunteers. The volunteers were healthy, not on any dietary restrictions, and had not taken antibiotics at least 3 months prior to donating. Fecal samples were collected by stool collection tubes and transferred to an anaerobic chamber within 30 min. Fresh fecal slurry (20 % w/v) was prepared in the phosphate buffered saline (PBS; pH 8.0) and homogenized using a high-speed stomacher (Seward, Worthing, UK) for 3 min at 300 rpm before inoculation into each fermentation test.

An anaerobic batch fermentation modified method from Charoensiddhi et al. (2016) was used to assess the effect of seaweeds (SW-U, SW-G) and their polysaccharide-enriched extracts (PF-U, PF-G) on fermentation characteristics and composition of gut microbiota. Fresh fecal samples were used as inoculum with 2.0 % (w/v) in each fermentation. Anaerobic conditions were maintained throughout the set-up of fermentations using an anaerobic chamber (Bactron IV Anaerobic Chamber Sheldon Manufacturing Inc., Cornelius, OR, USA).

Sample substrates at a concentration of 2.0 % (w/v) in fermentation media were used in each test. No substrate was used as the control or blank, and inulin (I2255, Sigma) at the same concentration of sample substrates was included as the positive control. Each fermentation was conducted in triplicate on each of three fecal donors. The fermentation medium modified from Charoensiddhi et al. (2016) contained the following (per L of distilled water): 2.0 g peptone, 2.0 g yeast extract, 2 g $NaHCO_3$, 0.5 g Bile salt, 0.5 g L-cysteine, 0.1 g NaCl, 0.05 g Hemin, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_20$, 0.01 g $CaCl_2.2H_20$, 10 μL Vitamin K, 2 mL Tween 80. The fermentation was maintained at 37 °C under an anaerobic condition for 24 h, pH 6.65–6.95 using pH controller adjusted by 0.5 M HCl and 0.5 M NaOH.

2.6. SCFAs, phenol, and p-cresol determination

The short chain fatty acids (SCFAs) analysis was performed by gas chromatography according to the method of Charoensiddhi et al. (2016) and McOrist et al. (2008) with slight modifications. 1 mL of fermentation samples were mixed thoroughly with 3 mL of internal standard solution (heptanoic acid 5.04 μmoL/mL of fermentation samples). Samples were vortexed and centrifuged at 2000g, 4 °C for 10 min, and 300 μL of supernatant was transferred to micro centrifuge tube, and then 10 μL of 1 M phosphoric acid was added to the supernatant. The supernatant was filtered through a nylon filter with a pore size of 0.45 μm, and 0.2 μL was injected into a Gas Chromatography equipped with Flame Ionisation Detector (GC-FID, model 7890A; Agilent Technologies, Santa Clara, CA, USA) and capillary column (DB-FFAP, 30 m × 0.53 mm × 0.5 μm). The initial oven temperature was held at 90 °C for 1 min and increased at 20 °C/min to 190 °C held for 2.5 min. The injector and detector temperatures were set at 210 °C. Helium was used as a carrier gas; the gas flow and septum purge rates were at 7.7 and 3.0 mL/min, respectively. A standard SCFAs mixture containing acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids, as well as the standard phenol and *p*-cresol were used for the calculation. The SCFAs concentrations were calculated in μmoL/mL. Quantification was performed based on the relative peak area of each SCFAs, phenol, and *p*-cresol external standard, adjusting the quantity of each compound based on that of the internal standard.

2.7. Gut microbiota analysis using 16 s rRNA next generation sequencing

The DNA extraction of fermentation samples was conducted using ZymoBIOMICS DNA Miniprep Kit: D4300 (Zymo research, Irvine, CA, USA) according to the manufacturer's instructions. The quantity and quality of extracted DNA samples were measured using a Nano Drop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), then stored at −20 °C prior to further analysis. The extracted DNA samples were amplified using PCR: Biometra *T*-gradient thermocycler (Biometra, Goettingen, Germany). The V3-V4 regions of the bacterial 16S rRNA gene sequences were amplified from the diluted DNA using universal primers; Macrogen Imina-V3-V4-F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG-3′ and Macrogen Imina-V3-V4-R 5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC TACTACHVG GGTATC TAATCC −3′ (Plupjeen et al., 2020). The PCR reaction mixture (50 μL) included 1 μL of DNA template (50 ng/μL), 1 μL of each primer (1 μM), 5 μL dNTPs (400 μM), 5 μL of 10x PCR buffer, 1 μL of i-Taq™ DNA Polymerase (0.1 unit) (iNtODEWORLD, Inc., MA, USA), and 36 μL of PCR water. Cycling parameters included the initial denaturation at 95 °C for 2 min, followed by 24 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s with a final extension step at 72 °C for 10 min. Reaction products were extracted from 1 % agarose gel by electrophoresis and subsequently purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

The gut microbiota was analyzed by MiSeq system (Illumina, USA) based on the modified method from Plupjeen et al. (2020). The obtained sequencing data were demultiplexed and quality-filtered using the Quantitative Insights into Microbial Ecology 2 platform (QIIME 2, Version 2019.1). The remaining high-quality reads were then used to identify chimeras, and the pooled high-quality reads were clustered into operational taxonomic units (OTUs). The number of reads obtained in this study was ~ 24,000 OTUs per sample. OTU taxonomic assignments were then generated using the Greengenes reference database (version gg_13_8_99) under a confidence threshold of 97 % (McDonald et al. 2012).

2.8. Statistical analyses

All measurements were performed in a triplicate independent analysis for each sample. Results are expressed as the means and standard deviations. The statistical analyses were performed using SPSS version 18.0 for Windows program (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the means, and differences were considered significant at $p < 0.05$ by Duncan's post-hoc tests.

The analysis of sequencing information was performed using R version 4.0.2 (2020–06-22) (64-bit) (R Core Team, 2020). Shapiro-Wilk test was performed to test the Normality distribution of data. The mean comparison was computed using ANOVA and Tukey analysis for normal distribution data. However, for the non-normal distributed data Kruskal wallis and Dunne test was applied with R Package dunn.test (Dinno, 2017) and FSA (Ogle et al., 2020). The non-parametric rank correlations were calculated by Kendall's tau correlation coefficient. The correlation analysis was performed using R Package ggpubr (Kassambara, 2020).

3. Results and discussion

3.1. Chemical compositions of seaweed materials

The compositions of *Ulva rigida* (SW-U) and *Gracilaria fisheri* (SW-G) were analyzed. The total dietary fiber, proteins, and minerals as ash were main components accounting for 43.28 % (soluble fiber 23.63 % and insoluble fiber 19.65 %), 32.34 %, and 19.06 % for *U. rigida* and 64.55 % (soluble fiber 42.21 % and insoluble fiber 22.34 %), 15.95 %, and 11.54 % for *G. fisheri* (based on DW). The similar sulfate content (~11 %) and low fat content (~2%) was observed in both seaweeds.

3.2. Characterization of seaweed polysaccharide-enriched extracts

The composition of the polysaccharide-enriched extracts from *U. rigida* (PF-U) and *G. fisheri* (PF-G) were characterized and shown in Table 1. The result showed that PF-U contained less carbohydrate and

Table 1
Chemical compositions of polysaccharide-enriched extracts of *U. rigida* (PF-U) and *G. fisheri* (PF-G).

Composition (%)	PF-U	PF-G
Carbohydrates	54.27 ± 8.58^{b}	77.36 ± 2.10^{a}
Uronic acid	31.84 ± 2.62	32.38 ± 0.61
Monosaccharide (% moL)		
- Fucose	0.18 ± 0.005^{a}	0.08 ± 0.01^{b}
- Arabinose	0.12 ± 0.02	-
- Rhamnose	63.79 ± 1.26	-
- Galactose	1.71 ± 0.46^{b}	65.05 ± 3.45^{a}
- Glucose	8.03 ± 0.23^{b}	30.81 ± 3.69^{a}
- Xylose	7.58 ± 0.34^{a}	2.23 ± 0.34^{b}
- Mannose	0.78 ± 0.38	-
- D-Glucuronic acid	17.81 ± 0.41^{a}	1.78 ± 0.21^{b}
Proteins	8.53 ± 0.25^{a}	3.21 ± 0.18^{b}
Sulfate	16.88 ± 0.99^{a}	12.20 ± 0.97^{b}
Molecular weight (kDa)	268.16	16.77

* Values are means ± SD (n = 3), except MW analyzed by HPGPC. Means in a row with different superscripts indicate significant differences ($p < 0.05$).

higher protein contents (54 % and 9 %, respectively) than PF-G (77 % and 3 %, respectively). The uronic acid content of both samples was similar (32 %). The macromolecular properties of polysaccharides are influenced by their primary structure, which is mainly determined by such as their sugar compositions, molecular weights (MW), and functional groups. In this study, both polysaccharides exhibited typical heteropolysaccharides. The PF-U consisted of rhamnose (64 %) and D-glucuronic acid (18 %) as a major neutral and acidic sugars with a minor of glucose and xylose with high MW (268 kDa). This may correspond to the ulvan backbone which is commonly made up of α- and β-(1,4)-linked monosaccharides (rhamnose, xylose, glucuronic acid, and iduronic acid) with characteristic repeating disaccharide units (Kidgell et al., 2019). In contrast, galactose (65 %) and glucose (31 %) were observed as main neutral sugars in the PF-G followed by a minor of xylose and D-glucuronic acid with lower MW (17 kDa). This may also correspond with the compositions of agarose and agaropectin which is the major components found in the agar, a complex mixture of red seaweed polysaccharides. Agarose is a neutral polysaccharide with a linear structure of repeating units of agarobiose formed by 3 linked β-D-galactose and 4 linked 3,6-anhydro-α-L-galactose, while agaropectin is an acid polysaccharide containing sulphate groups, pyruvic acid, and D-glucoronic acid conjugated to agarobiose (Duckworth and Yaphe, 1971). The sulfated content of PF-U (17 %) and PF-G (12 %) were rather high relative to polysaccharide extracts from other *Ulva* (~15.5 % sulfate) and *Gracilaria* (7–9 % sulfate) species (Kidgell et al., 2019; Khan et al., 2019; Khan et al., 2021). The potential health benefits of sulfated polysaccharides have been demonstrated for many beneficial biological activities such as anticoagulation, antioxidation, immune regulation, anti-tumorigenesis, and metabolic modulation (Huang et al., 2022).

FTIR is an effective method to identify the characteristic structure of polysaccharides. The FTIR spectrum of the SW-U, PF-U, SW-G, and PF-G is shown in Fig. 1a-d. For SW-U (Fig. 1a) and PF-U (Fig. 1b) spectrum, the important absorptions were at approximately 1254 cm^{-1} and 1030 cm^{-1}, which are considered as the fingerprint region for ulvan (Morelli et al., 2016). Absorption at 1254 cm^{-1} was characteristic of stretching vibration of sulfate ester (S═O), while a band at 1030 cm^{-1} referred to C—O stretching of the two main sugars, rhamnose and uronic acid (Yaich et al., 2017). The above result was in agreement with carbohydrate compositions of the PF-U (Table 2). The uronic acid (32 %) and the rhamnose (64 %) were the main constituents in the PF-U. The signals at 850 and 796 cm^{-1} were the bending vibration of C—O—S of the sulfate groups in the axial and equatorial position, respectively (Chi et al., 2020). This result indicated that the SW-U and PF-U probably contain more than one type of sulfate group. Different FTIR spectra of SW-G (Fig. 1c) and PF-G (Fig. 1d) were observed compared to SW-U and PF-U due to their different major polysaccharide components. The absorption bands at 1372 and 1254 cm^{-1} were the vibration of sulfate groups (Guerrero et al., 2014). The band at 1160 cm^{-1} was the vibration of ester sulfate, and the small signal at 850 cm^{-1} was assigned to the sulfate in C-4 from galactose indicating the presence of sulfation on C4 of β-D-galactopyranosyl (Guerrero et al., 2014). The intense band observed at approximately 1088 cm^{-1} was equivalent to the skeleton of galactans (Melo et al., 2002). In addition, the region at 930 cm^{-1} was attributed to the C—O—C group of 3,6-anhydrogalactose (3,6-AG), representing the agar family (Yarnpakdee et al., 2015). The band at 891 cm^{-1} was attributed to β-D-galactose units in agar (Rajasulochana and Gunasekaran, 2009). Importantly, the similar spectrum between SW-U and PF-U, also SW-G and PF-G was observed, so it may infer that the extraction procedures can maintain ulvan and agar structures and consequently their intrinsic properties.

3.3. *In vitro digestibility*

The digestibility of samples was determined using an *in vitro* human digestion model in order to understand the likelihood of components reaching the large bowel and the resident microbiota. The reducing sugar release of PF-U was not significantly different ($p > 0.05$) among the oral, gastric, and intestine phases compared to the undigested sample (Fig. 2a). Also, there was a very low concentration of reducing sugar (<2.5 mg/mL) released from PF-G during the oral and gastric phases, but no significant difference ($p > 0.05$) was observed between the intestine phase and undigested sample. After passing the gastrointestinal tract model, the particle size of both seaweed polysaccharide-enriched extracts was not significantly different ($p > 0.05$) (Fig. 2b).

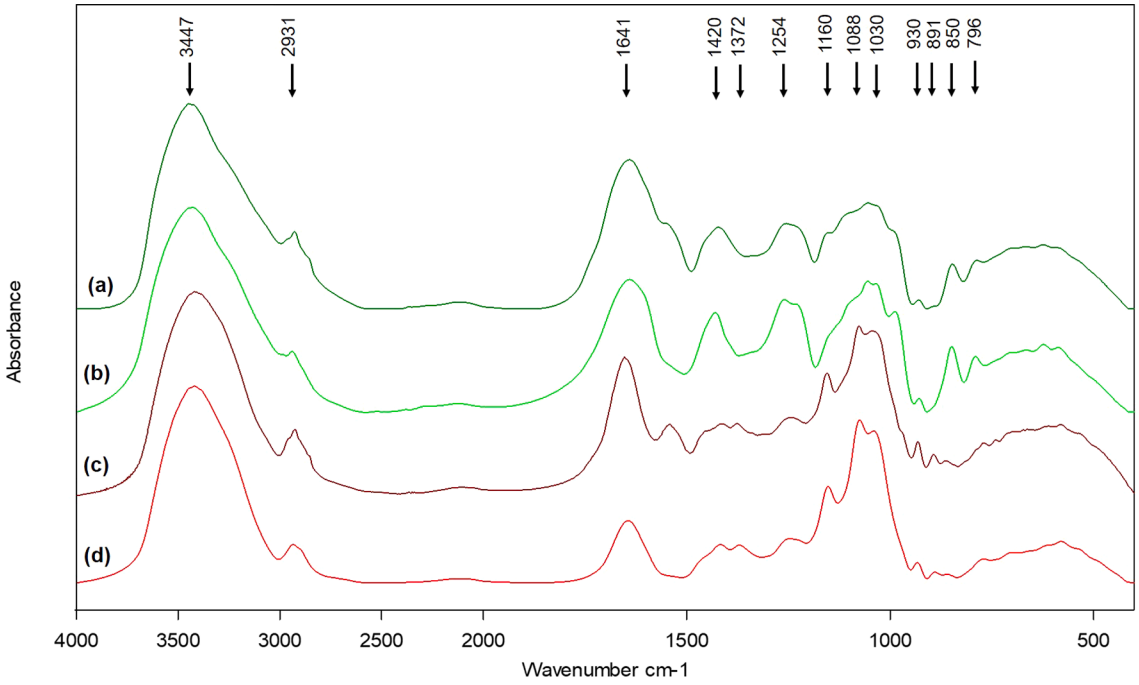

Fig. 1. FT-IR spectra of (a) Seaweed *U. rigida* (SW-U), (b) Polysaccharide-enriched extracts from *U. rigida* (PF-U), (c) Seaweed *G. fisheri* (SW-G), and (d) Polysaccharide-enriched extracts from *G. fisheri* (PF-G).

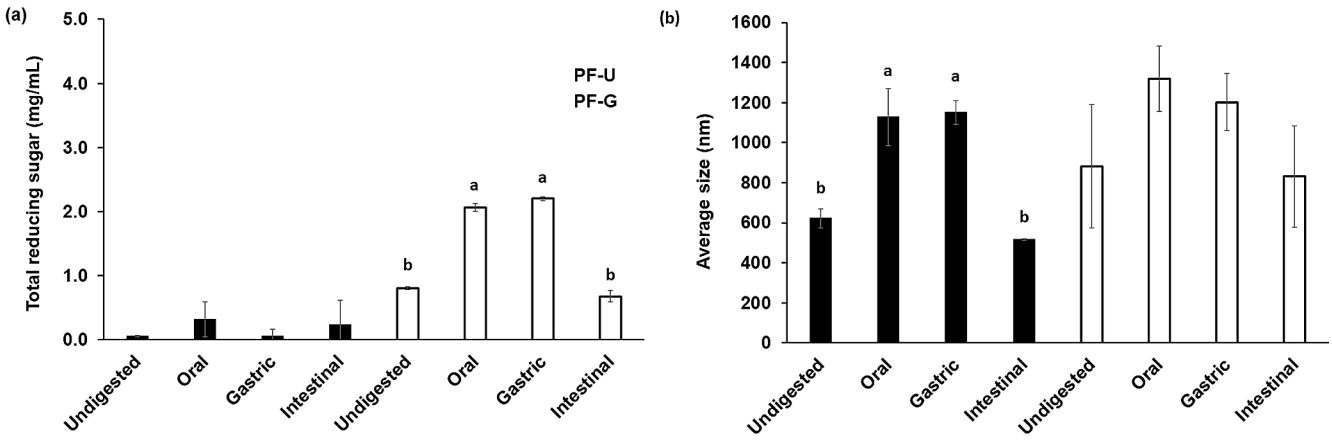

Fig. 2. (a) Reducing sugar content and (b) Particle size (polydispersity index ~ 0.4–0.9) of polysaccharide-enriched extracts from *U. rigida* (PF-U) and *G. fisheri* (PF-G) during different phases of a multistage gastrointestinal tract digestion model. Values are means ± SD (n = 3) with different letters are significantly different ($p < 0.05$).

However, an increase in the size during the oral and gastric phases might be due to the addition of enzymes and the change of polysaccharide conformation. In addition, the different conformation of both seaweed polysaccharide-enriched extracts in each digestion phase using SEM was observed in Fig. 3. Therefore, it can be concluded that both seaweed polysaccharide-enriched extracts were not digested, but the morphology slightly change during passing through the gastrointestinal tract model.

3.4. *In vitro human fecal fermentation*

The health benefits of seaweed and seaweed polysaccharide-enriched extracts were assessed by including them in an *in vitro* anaerobic batch fermentation system containing human fecal inocula that mimic the environment of the human large bowel. The production of key beneficial gut fermentation products, short-chain fatty acids (SCFAs), and the growth and activities of certain gut microorganisms, are used as indicators of potential benefits.

3.4.1. *SCFAs production*

The concentrations of SCFAs are considered as important reflections of beneficial microbial activity (Fernández et al., 2016). Concentrations of acetic acid, propionic acid, and butyric acid increased, with the highest concentrations observed at 24 h fermentations in each case. Acetic and propionic acids were the predominant SCFAs produced in all fermentations. At 24 h of fermentation, the results from three donors (Fig. 4) showed that total SCFAs concentrations of fermentations with all seaweeds and polysaccharide-enriched extracts (29.4–35.4 μmoL/mL) were significantly higher ($p < 0.05$) than the control (17.9 μmoL/mL), but significantly lower ($p < 0.05$) than the positive control inulin (102.3 μmoL/mL). These results indicated that the inulin, a well-known prebiotic, is fermented by human gut microbiota more easily than seaweeds and their polysaccharide-enriched extracts. This might be due to the low MW of inulin (2.12 kDa), which can be classified as oligosaccharides, while seaweeds and their extracts contained high MW polysaccharides (PF-G 16.77 kDa and PF-U 268.16 kDa). SCFAs concentrations of fermentations with SW-G and PF-G tended to be higher compared to the SW-U and PF-U. It was interesting that the fermentation with PF-G increased butyric acid production to levels comparable to the inulin (5.4 and 6.9 μmoL/mL), and significantly higher ($p < 0.05$) than for other treatments (1.8–3.3 μmoL/mL). This observation probably resulted from the different compositions and characteristics of both seaweeds and polysaccharide-enriched extracts. The higher dietary fiber of SW-G (65 %) and higher soluble polysaccharides of PF-G (galactose as key sugar component) with lower MW (Table 1) may be mainly attributed to

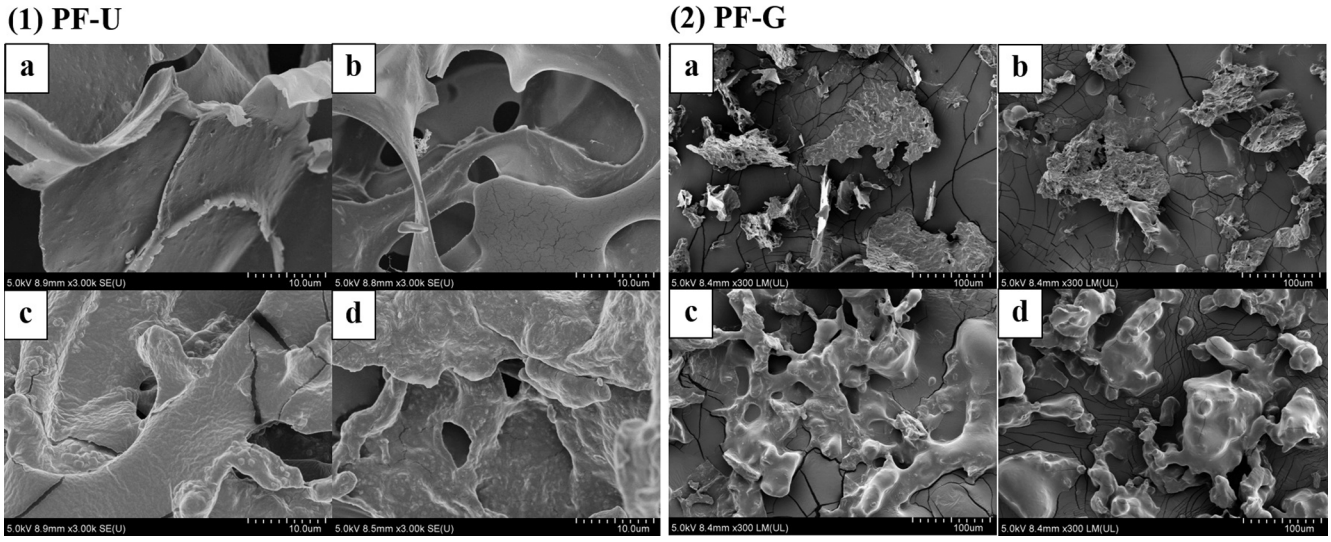

Fig. 3. Morphology of (1) Polysaccharide-enriched extracts from *U. rigida* (PF-U) under SEM (3000X) and (2) Polysaccharide-enriched extracts from *G. fisheri* (PF-G) under SEM (300X) of (a) Undigested sample and samples after (b) Oral phase, (c) Gastric phase, and (d) Intestine phase.

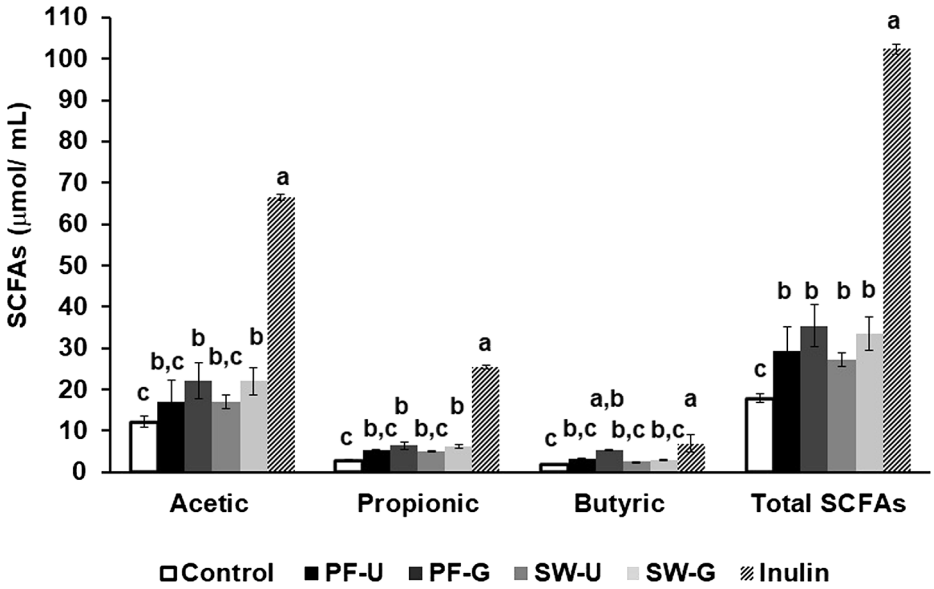

Fig. 4. Comparison of SCFAs concentrations following 24 h fecal fermentations of polysaccharide-enriched extracts of *U. rigida* (PF-U) and *G. fisheri* (PF-G), dried seaweeds *U. rigida* (SW-U) and *G. fisheri* (SW-G), inulin, and no added substrate (control). Values are means ± SD (n = 3), means with different letters in the same SCFAs are significantly different ($p < 0.05$).

the fermentation by intestinal bacteria, eventually accelerating the production of SCFAs. Parkar et al. (2021) reported that the fiber-derived sugar composition in foods may influence gut microbiome structure and metabolic activity. Additionally, it has been recognized recently that SCFAs are important link between microbiota and the immune system by modulating different aspects such as development of intestinal epithelial cells and leukocytes, survival and function through activation of G protein coupled receptors (FFAR2, FFAR3, GPR109a, and Olfr78), and modulation of the activity of enzymes and transcription factors including the histone acetyltransferase and deacetylase and the hypoxia-inducible factor (Corrêa-Oliveira et al., 2016). It was also noted that all samples produced low levels of isobutyric, valeric, isovaleric, and caproic acids, as well as phenols and *p*-cresol (data not shown). Phenol and *p*-cresol are an excessive production of microbial products derived from the protein fermentation. The accumulation of these compounds in the gut are associated with the risk of bowel diseases and the loss of gut integrity and function (Windey et al., 2012).

3.4.2. Gut microbiota community

This study explored the impact of seaweeds and their polysaccharide-enriched extracts on the composition of gut microbiota by 16S rRNA sequencing using human fecal batch fermentation samples compared to those on commercial inulin and control or blank. The taxonomic compositions of the tested samples at the phylum level are shown in Fig. 5a. The shade of color indicates the relative abundance. The higher relative abundance shows in the red, orange, yellow, white, grey, light blue, and dark blue color, respectively. Firmicutes, Proteobacteria, and Bacteroidetes were the dominant bacterial phyla. Rinninella et al. (2019) reported that apart from the dominant Firmicutes and Bacteroidetes, bacterial phyla within the healthy colonic microbiota include Proteobacteria, Actinobacteria, and Verrucomicrobia. The ratio of Firmicutes to Bacteroidetes is considered to be one of the biological indicators of obesity (Mathur and Barlow, 2015; Di et al., 2018). In our study, the percentage of relative abundance ratio of Firmicutes to Bacteroidetes decreased in the PF-U (8.32), PF-G (11.98), SW-G (14.70), and inulin (4.65) fermentations compared to the control or blank (20.28), suggested that the *G. fisheri*, both polysaccharide-enriched extracts, and inulin might be associated with a reduced risk of obesity or excessive body weight. However, this correlation must be further verified in an *in vivo* study. An increase in the relative abundance of Proteobacteria was observed in the fermentations supplemented with seaweeds and polysaccharide-enriched extracts, except for SW-G, but not statistically significant ($p > 0.05$).

The microbial growth stimulations by the seaweeds and their polysaccharide-enriched extracts were observed in some particular groups of microorganisms as compared to the control. The relative abundance of *Lachnospiraceae* increased when the SW-G were supplemented (56.4 % vs control 50.1 %) (Fig. 5b). Also, the proportion of *Ruminococcaceae* was higher in the PF-U (20.0 %) and SW-G (10.1 %) supplemented conditions in comparison with the control group (6.9 %). The *Lachnospiraceae* and *Ruminococcaceae* are the two most abundant families in the order *Clostridiales* in phylum Firmicutes. *Lachnospiraceae* has been associated with butyrate production, which is important for the health of colonic epithelial tissue, and some *Ruminococcaceae* has been shown to deplete Crohn's disease (Biddle et al., 2013). Menni et al. (2017) also reported that *Ruminococcaceae* may be functionally linked to a lean phenotype and nominally protective of weight gain. This was in agreement with our study, the significant increase ($p < 0.05$) in *Roseburia* and *Faecalibacterium* shown to be selectively promoted by SW-G (2.5 % vs control 0.5 %) (Fig. 5c). The abundance of *Blautia* increased when the PF-G and SW-G were supplemented (30.7 % vs control 18.6 %). This gut microbial genus is known as the butyric and acetic acid producer which decreases obesity by regulating G-protein coupled receptors (GPR) 41 and 43 (Ozato et al., 2019). Additionally, the increased levels of *Bacteroidaceae* or *Bacteroides* observed in the PF-G and SW-G could be due to their ability to utilize seaweed polysaccharides as these bacteria possess large amounts of carbohydrate active enzymes (CAZymes). Studies have found that CAZymes can degrade marine algae polysaccharides such as fucoidan, laminaran, alginate, carrageenan, and agar into oligomers (Hehemann et al., 2014). The high levels of *Bifidobacteriaceae*, which are known markers of prebiosis, and *Prevotellaceae* were found in only the fermentation with inulin (a positive control in this study). Flint et al. (2015) reported that fecal microbiota with high *Prevotella* was observed in adults who consume a diet rich in fiber. It was notice that apart from polysaccharides, other seaweed-derived components including polyphenols and peptides could modulate the abundance and diversity of beneficial gut microbiota (Shannon et al., 2021). Therefore, further studies will be necessary to determine the potential prebiotic effects of other components in *U. rigida* and *G. fisheri* besides polysaccharides.

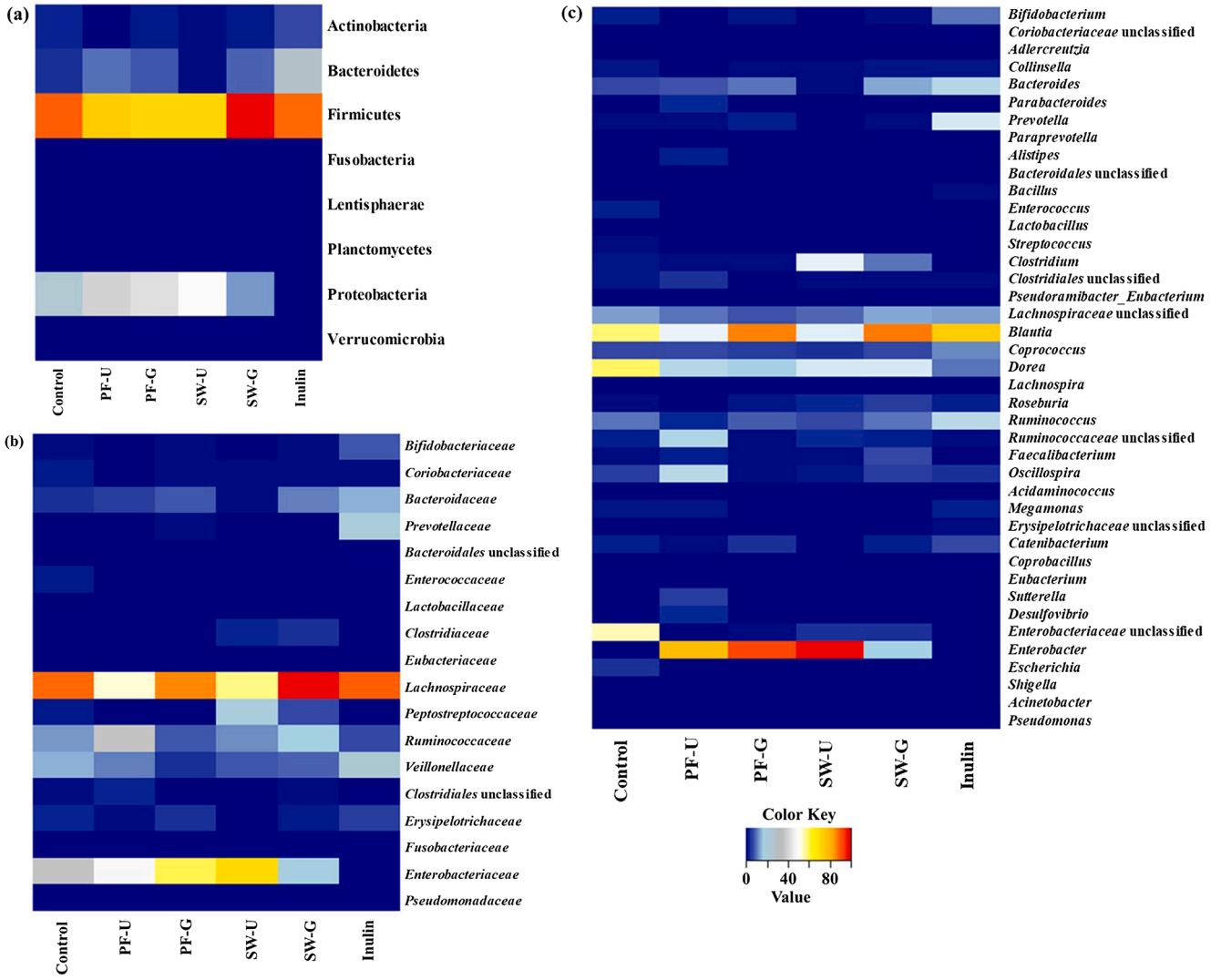

Fig. 5. A heatmap displaying the normalized bacterial relative abundance of (a) phylum (b) family, and (c) genus levels following 24 h fecal fermentations of polysaccharide-enriched extracts of *U. rigida* (PF-U) and *G. fisheri* (PF-G), dried seaweeds *U. rigida* (SW-U) and *G. fisheri* (SW-G), inulin, and no added substrate (control). In normalization, zero percent is defined as the smallest value, while one hundred percent is defined as the largest value in each dataset.

Apart from the changes of beneficial bacteria, the potential pathogenic bacteria were observed. Relative to the control, the low abundance of *Enterobacteriaceae*, which are Gram-negative bacteria was especially found in the fermentations with SW-G and inulin (10.3 % and 0.3 % vs control 19.8 %). Significantly lower abundances ($p < 0.01$) of *Escherichia coli* were observed following fermentation with seaweeds, polysaccharide-enriched extracts, and inulin. The same result was observed with bacterial genus *Dorea* which has been linked with inflammatory diseases such as Crohn's disease (Shahi et al., 2017). However, an increase in the relative abundance of *Enterobacter* was observed in the fermentations supplemented with especially SW-U compared to the control. The higher level of *Peptostreptococcaceae*, a family within the order *Clostridiales*, was observed for SW-U fermentations (Fig. 5c). This bacterium appears to be over-represented in the gut of colorectal cancer patients (Ahn et al., 2013). A lower relative abundance of *Veillonellaceae* in the fermentations with seaweeds and their polysaccharide-enriched extracts (2.8–6.1 %) was found compared to the positive control inulin (13.2 %). Some studies demonstrated the abundance of bacteria in the family *Veillonellaceae* has been shown to be significantly decreased by a gluten-free diet, and an increase in its abundance was consistently reported in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and cirrhosis patients, suggesting pro-inflammatory activities (Bonder et al., 2016).

Correlations occurring between gut microbiota and SCFAs concentrations were also observed and shown in Fig. 6. Results showed that *Bifidobacterium*, *Bacteroides*, *Prevotella*, *Bacillus,* and *Ruminococcus* correlated positively with total SCFAs. Within these bacteria, *Bifidobacterium* correlated positively with acetic, propionic, and caproic acids, while *Bacteroides* related to propionic and isovaleric acid production. *Prevotella* and *Bacillus* correlated positively with all three main SCFAs and caproic acid, only isovaleric related to *Prevotella*. *Ruminococcus* correlated positively with acetic and butyric acid production. Supporting these findings, Flint et al. (2015) reported that *Firmicutes*, in particular *Clostridium* Clusters IV and XIVa (including *Faecalibacterium*, *Eubacterium*, *Roseburia*, and *Ruminococcus*) and *Bacillus*, have been identified as the dominant producers of butyrate in the colon. *Bifidobacterium* species ferment sugars and yield mainly acetate and lactate. However, the production of SCFAs may involve bacterial cross-feeding amongst microorganisms during the breakdown of complex carbohydrates (Ríos-Covián et al., 2016). The negative correlations between potentially pathogenic microbes and SCFAs concentration were also found, in particular to bacteria of the genus *Dorea*.

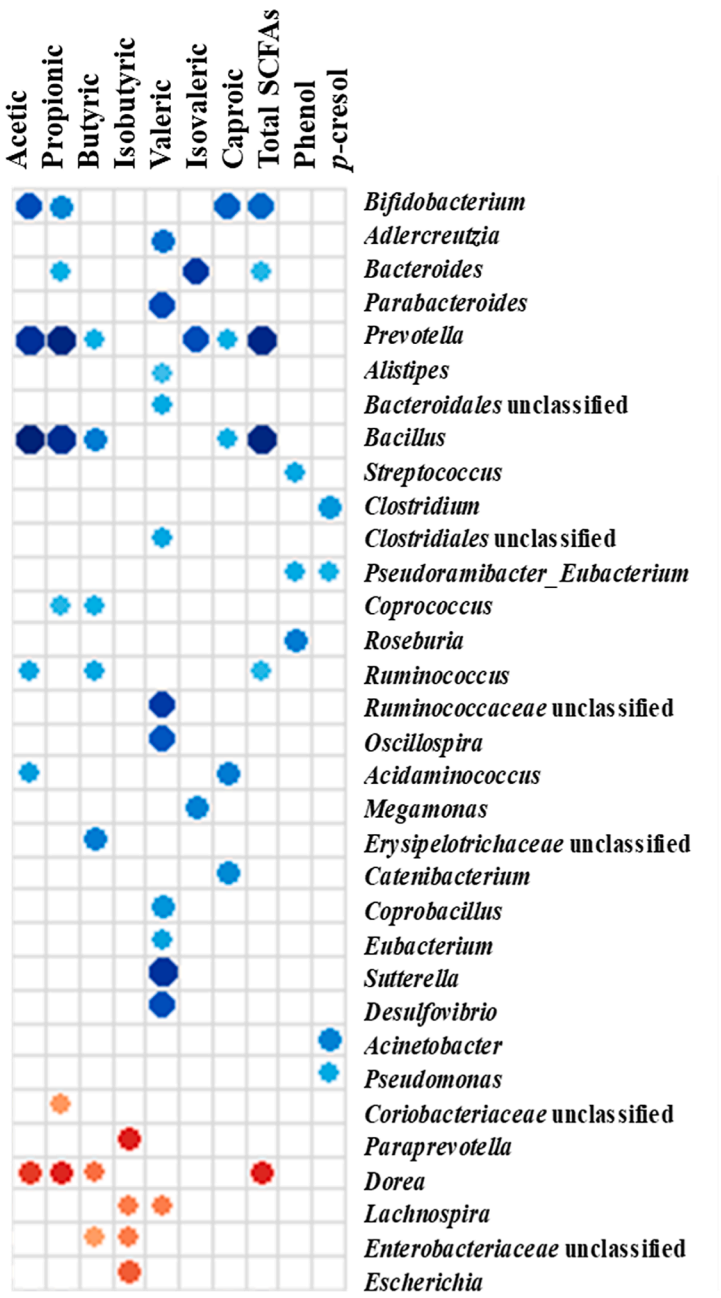

Fig. 6. Correlations occurring between gut microbiota and SCFAs concentrations following 24 h fecal fermentations of seaweeds and their polysaccharide-enriched extracts. Blue circles represent significant ($p < 0.05$) positive correlations. Red circles represent significant ($p < 0.05$) negative correlations. Darker colors represent stronger correlation. Non-significant correlations are not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The present study reported new information on the potential gut health benefits of the green seaweed *Ulva rigida* (SW-U) and the red seaweed *Gracilaria fisheri* (SW-G), also their polysaccharide-enriched extracts (PF-U and PF-G, respectively) through *in vitro* digestion and *in vitro* human fecal fermentation. Both PF-U and PF-G were not digested, but its morphology was changed. Seaweeds and their polysaccharide-enriched extracts were degraded by gut microbiota, resulting in a high level of SCFAs with acetic and propionic acids being the predominant metabolites. Especially, SW-G and PF-G tended to be higher in the production of SCFAs than the SW-U and PF-U. Fermentations of SW-G increased the diversity of beneficial bacteria including *Roseburia* and *Faecalibacterium*. The different compositions, particularly the type, level of soluble polysaccharides, and MW are most likely to be responsible for any differences in potential benefits. The SW-G and PF-G show promise for development as functional food ingredients and dietary supplements with gut health benefits based on the ability to boost SCFAs production and stimulate growth of beneficial gut microbes. Further investigations in an *in vivo* are required to substantiate the prebiotic potential of these seaweed and their polysaccharide-enriched extracts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors gratefully acknowledge the funding support from the Thailand Research Fund (TRF) grant number MRG6280033, and the seaweed material support from Phetchaburi Coastal Aquaculture Research and Development Center. The authors also thank for the

technical support from Dr. Jiratthitikan Sriprablom and Dr. Siriluck Wattanavanitchakorn.

Ethics ***Statement***

All feces-related experimental work was approved by the Human Research Ethics Approval.

Committee of the Kasetsart University (Approval title: Impact of Thai seaweed extracts on gastrointestinal digestibility and health benefits; KUREC-HS61/040).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105204.

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