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Comparative analysis of prebiotic effects of seaweed polysaccharides laminaran, porphyran, and ulvan using *in vitro* human fecal fermentation

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ABSTRACT

Laminaran, porphyran, and ulvan are major seaweed polysaccharides in brown, red, and green algae, respectively. We compared their prebiotic effects using individual microbial fermentability test and *in vitro* fecal fermentation. The fermentability test showed that these polysaccharides were selectively utilized by Bifidobacteria, Lactobacilli, and Bacteroides ($\Delta OD_{580\ nm}$, 0.2–1.0), while no growth of harmful bacteria was observed. *In vitro* fecal fermentation for 24 h showed growth stimulation effect of laminaran on Bifidobacteria (Δ8.3%/total bacteria) and Bacteroides (Δ13.8%/total bacteria) promoting the production of acetate and propionate. Ulvan exhibited same result on Bifidobacteria (Δ8.5%/total bacteria) and Lactobacillus (Δ6.8%/total bacteria) promoting the production of lactate and acetate; however, porphyran showed little prebiotic effect. Laminaran was fermented slowly compared to fructooligosaccharides and this may permit production of short-chain fatty acids in distal colon. This *in vitro* study demonstrates that the seaweed polysaccharides tested, particularly laminaran and ulvan, have prebiotic effects on microbiota in human colon.

1. Introduction

There is an increasing body of evidence that the consumption of algal food products may have health and nutritional benefits (Wells et al., 2016). Seaweeds or marine macroalgae are one of nature's most biologically active resources. The compounds obtained from them show a wealth of bioactivities, such as antibacterial activity of *Laminaria* spp. (Chandini, Ganesan, & Bhaskar, 2008; Yuan & Walsh, 2006), anti-inflammatory properties of *Sargassum fulvellum* (Kang et al., 2008), anti-coagulant activity of *Lomentaria catenata* (Pushpamali et al., 2008), anti-viral activity of *Sargassum tenerrimum* (Sinha, Astani, Ghosh, Schnitzler, & Ray, 2010), and apoptotic activity of *Porphyra* spp. (Kwon & Nam, 2006). Therefore, seaweed-derived compounds have important applications in a range of food, pharmaceutical, and cosmetic products (D'Ayala, Malinconico, & Laurienzo, 2008; Dhargalkar & Verlecar, 2009). In addition to bioactive components, seaweeds are a rich source of dietary fiber (25–75% dry weight), of which water-soluble fiber constitutes approximately 50–85% (Jiménez-Escrig & Sánchez-Muniz, 2000). Seaweeds are commonly classified into three main groups based on their pigmentation. Phaeophyta, or brown seaweeds, are predominantly brown due to the presence of the carotenoid fucoxanthin, and the primary polysaccharides present include alginates, laminarans, fucans, and cellulose (Goni, Valdivieso, & Gudiel-Urbano, 2002). *Pyropia* and *Porphyra* are red algae and porphyran is a sulfated polysaccharide extracted from *Porphyra* (Zhang et al., 2010). *Chlorophyta* or green seaweeds are dominated by chlorophyll *a* and *b*, with ulvan being the major polysaccharide component (Robic, Rondeau-Mouro, Sassi, Lerat, & Lahaye, 2009).

The molecule laminaran, also known as laminarin is a storage glucan found in brown algae; first discovered in *Laminaria* species. The chemical structure of laminaran consists mainly of a linear β-(1-3)-linked glucan with some random β-(1-6)-linked side-chains depending on the variety of seaweed (Brown & Gordon, 2005; Yun, Estrada, Van Kessel, Park, & Laarveld, 2003). It was reported that laminaran has antitumor, anti-inflammatory, anticoagulant, and antioxidant activities (Kadam, Tiwari, & O'Donnell, 2015). Porphyran has a linear backbone consisting of 3-linked β-D-galactosyl units alternating with either 4-linked alpha-L-galactosyl 6-sulfate or 3,6-anhydro-alpha-L-galactosyl units. The composition includes 6-*O*-sulfated L-galactose, 6-*O*-methylated D-galactose, L-galactose, 3,6-anhydro-L-galactose, 6-*O*-methyl D-galactose, and ester sulfate. Some of the esters are present as 1-4-linked L-galactose 6-sulfate (Zhang et al., 2005). Because of its high content in *Pyropia*, the nutritional and biological significance of *Pyropia* has attracted much interest. The antitumor (Kwon & Nam, 2006),

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antioxidative (Isaka et al., 2015; Zhang et al., 2009), antihyperlipidemic (Wang et al., 2017), neuroprotective (Liu, Deng, Geng, Wang, & Zhang , 2019) and immuno-modulating (Bhatia et al., 2013; Isaka et al., 2015) activities of porphyran have been well studied. Ulvan, obtained from green seaweed of the *Ulva* genus, is composed of sulfated L-rhamnose, D-glucuronic acid, and its C5-epimer L-iduronic acid, and a minor fraction of D-xylose (Lahaye & Robic, 2007; McKinnel & Percival, 1962). The three main repetitive units are α-L-rhamnose-3-sulfate-1,4-β-D-glucuronic acid (ulvanobiouronic acid A), α-L-rhamnose-3-sulfate-1,4-α-D-iduronic (ulvanobiouronic- acid B), and α-L-rhamnose-3-sulfate-1,4-β-D-xylose (Lahaye & Robic, 2007). Antioxidant and immunomodulatory activities of ulvan were reported recently (Berri et al., 2017; Rahimi, Tabarsa, & Rezaei, 2016).

At present, significant attention is being paid to the use of polysaccharides from seaweeds as prebiotics, which cannot be decomposed by the enzymes of the upper gastrointestinal tract and serve as an important source of dietary fibers (Koneva, 2009). Han et al. (2019) reported prebiotic effects of three marine algae oligosaccharide including alginate oligosaccharides, agarose oligosaccharides, and κ-carrageenan oligosaccharides evaluated by *in vitro* fermentation. In addition, polysaccharides extracted from brown seaweed *Ecklonia radiate* was reported to exhibit prebiotic effects showing enhanced short chain fatty acids (SCFAs) production and commensal bacterial genera in *in vitro* fermentation (Charoensiddhi, Conlon, Vuaran, Franco, & Zhang, 2016). Although previous studies have reported the prebiotic effect of seaweed polysaccharides, only individual polysaccharides have been studied, and there is very little research on ulvan, a rare polysaccharide that is often difficult to ingest. In addition, there are few studies on the fermentability of the seaweed polysaccharides by individual human fecal microorganism comprising beneficial/commensal or harmful bacteria.

Therefore, the aim of this study was to compare the prebiotic effect of three seaweed polysaccharides, laminaran, porphyran, and ulvan, by employing the fermentability test using individual intestinal bacteria as well as the *in vitro* human fecal fermentation system. For this purpose, the fermentability test was carried out by individual bacterial cultivation, and bifidogenic effects with production of SCFAs were analyzed by pH-controlled human fecal fermenter system and HPLC system, respectively. SCFAs are particularly important for colon health because they are the primary energy source for colonic cells and the signal compounds that induce anti-carcinogenic and anti-inflammatory activities (Koh, De Vadder, Kovatcheva-Datchary, & Backhed, 2016; Walker, Duncan, Leitch, Child, & Flint, 2005).

2. Materials and methods

2.1. Materials

Seaweed polysaccharides used in the experiment were porphyran (purity ≥ 80%, Carbosynth, UK), laminaran (purity ≥ 85%, Carbosynth, UK), and ulvan (purity ≥ 80%, Elicityl, France). To obtain pure polysaccharide fractions, the powder was solubilized in 80% ethanol at 80 °C for 2 h and centrifuged at 4000*g* for 15 min to remove the supernatant containing mono-, di-, and oligosaccharides. The precipitated samples were dried in a cool and ventilated place under N_2 gas. Fructooligosaccharide (FOS) and vitamin K_1 were purchased from Wako (Osaka, Japan), peptone water and yeast extract were from BD (Franklin Lakes, NJ, USA), $CaCl_2{\cdot}2H_2O$, K_2HPO_4, KH_2PO_4, and NaCl were from Junsei (Tokyo, Japan), bile salts, L-cysteine hydrochloride, hemin, $MgSO_4{\cdot}7H_2O$, $NaHCO_3$, and resazurin solution were from Sigma Aldrich (St. Louis, MO, USA), and Tween 80 was from VWR (Radnor, PA, USA); all chemicals were of analytical grade.

2.2. Utilization of seaweed polysaccharides by individual bacterial species

Bacterial strains used in this study are listed in Table 1 and they were obtained from American Type Culture Collection (ATCC, Manassas, USA) or from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). To study the growth of individual bacterial strains in the presence of seaweed polysaccharides, sugar-free basal medium (BHI) was prepared according to an established protocol (Zhang, Mills, & Block, 2009). Seed cultures were incubated overnight until the optical density at 580 nm reached 0.5. Seaweed polysaccharides (0.2%, w/v) were dissolved in BHI medium for 1 h before inoculation with 1% (w/v) bacterial preculture in PBS. Fermentation was conducted under anaerobic conditions at 37 °C and microbial growth was measured in a microtiter plate at 580 nm.

Table 1
Strains used in this study.

Species	Origin
Lactobacillus brevis ATCC 14869^T	Fecal, human
Lactobacillus acidophilus ATCC 4356^T	Fecal, human
Lactobacillus reuteri ATCC 23272^T	Fecal, human
Lactobacillus rhamnosus ATCC 53103	Fecal, human
Lactobacillus plantarum ATCC 10241	Fecal, human
Weissella confusa KACC 11841^T	Fecal, human
Weissella cibaria KACC11845	Kimchi
Enterococcus faecalis ATCC 29212	Urine, human
Bifidobacterium breve ATCC 15700^T	Fecal, human
Bifidobacterium longum ATCC 15707^T	Fecal, human
Bifidobacterium bifidum ATCC 29521^T	Fecal, human
Bacteroides fragilis ATCC 25285^T	Appendix abscess
Bacteroides vulgatus ATCC 8482^T	Fecal, human
Bacteroides ovatus ATCC 8483^T	Fecal, human
Bacteroides dorei DSM 17855^T	Fecal, human
Bacteroides thetaiotaomicron ATCC 29148^T	Fecal, human
Bacteroides uniformis ATCC 8482	Fecal, human
Clostridium perfringens ATCC 13124^T	Bovine
Bacillus cereus ATCC 10876	Contaminated flask
Escherichia coli ATCC 11775^T	Urine
Listeria monocytogenes ATCC 19115	Fecal, human
Salmonella Typhimurium ATCC 14028	Tissue, chickens
Staphylococcus aureus ATCC 6538	Fecal, human

The superscripted T indicates type strain.

2.3. In vitro fecal fermentation

In vitro human fecal fermentation of seaweed polysaccharides was conducted according to an established protocol (Mandalari, Nueno-Palop, Bisignano, Wickham, & Narbad, 2008). Water-jacketed fermenter vessels (300 mL) were filled with 135 mL of pre-sterilized basal growth medium (2 g/L peptone water, 1 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $CaCl_2{\cdot}2H_2O$, 2 g/L $NaHCO_3$, 0.5 g/L bile salts, 0.5 g/L L-cysteine hydrochloride, 50 mg/L hemin, 10 μL/L vitamin K_1, 2 mL/L Tween 80). They were inoculated with 15 mL of fecal slurry (10% w/v), prepared by mixing and homogenizing freshly voided adult feces in 0.1 M PBS at pH 7.0. Fresh fecal samples were collected from 5 healthy adults who had not received antibiotics or pre/probiotics and had no recent history of gastrointestinal disorders. The study protocol and consent forms were approved by the Institutional Review Board of Chungbuk National University (CBNU-201311-BR-022-01). Experiments were initiated within two hours of initial stool collection to ensure that the fecal microbiota were representative of fresh fecal and, therefore, of colon microbiota. Seaweed polysaccharides and FOS were added to a final concentration of 1% (w/v). Slurry in each vessel was magnetically stirred, and the pH and the temperature was maintained at pH 6.8 and at 37 °C, respectively. Anaerobic conditions were maintained by sparging the vessels with oxygen-free nitrogen gas at a flow rate of 15 mL/min. Resazurin (1 mg/L) was added to the medium as an indicator of anaerobic conditions. Samples (5 mL) were removed at 12 h and 24 h for the enumeration of bacteria and SCFAs analysis. For the enumeration of intestinal bacteria, quantitative real-time polymerase chain

Table 2
Genus-specific primers used in this study.

Target genus	Primers	Sequence (5′–3′)
Bifidobacteria	F-bifido	CGC GTC YGG TGT GAA AG
	R-bifido	CCC CAC ATC CAG CAT CCA
Lactobacilli	Lacto-F	AGC AGT AGG GAA TCT TCC A
	Lacto-R	CAC CGC TAC ACA TGG AG
Bacteroides	Bact934F	GGA RCA TGT GGT TTA ATT CGA TGA T
	Bact1060R	AGC TGA CGA CAA CCA TGC AG
Clostridium	g-Ccoc-F	AAA TGA CGG TAC CTG ACT AA
	g-Ccoc-R	CTT TGA GTT TCA TTC TTG CGA A
Total bacteria	TBA-F	CGG CAA CGA GCG CAA CCC
	TBA-R	CCA TTG TAG CAC GTG TGT AGC C

reaction (qPCR) was conducted using genus-specific primer sets (Table 2) according to previous study (Moon et al., 2015; Vigsnæs, Holck, Meyer, & Licht, 2011).

2.4. Monitoring of microbial changes by real-time PCR assay

DNA was extracted from each of fermentation samples using the DNA extraction kit for bacteria (Biofact, Deajeon, Korea), following the manufacturer's protocol. The purified DNA was stored at −20 °C until use. Amplification and detection of purified bacterial DNA by real-time PCR was performed with the IQ™5 from Bio-Rad (Hercules, CA, USA) using optical grade 96-well plates. Each amplification reaction was carried out in duplicate for each of the fermentation samples. The final reaction volume was 20 μL, containing 10 μL 2× SYBR Green Supermix qPCR (Bioneer, Korea), 1 μL of the primers (10 pmole/L), 1 μL template DNA (5 ng/L), and nuclease-free water (Qiagen, Hilden, Germany). The amplification program consisted of one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The relative quantities and relative ratios of gene targets encoding 16S rRNA gene sequences of the bacterial taxa were calculated using $E\Delta^{CT}$ and $E\Delta^{CT\ (\text{fermentation sample})}/E\Delta^{CT\ (\text{original bacterial community})}$, respectively, where E is the efficiency of the primer calculated from the slope of the standard curve ($E = 10^{-1/\text{slope}}$), and ΔCT is the CT value of the bacterial target normalized against the CT value of the total bacterial population in the sample (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007).

2.5. Chemical analysis

Samples collected from the culture were frozen in liquid nitrogen until analysis. After thawing, the samples were centrifuged at 16,000*g* for 10 min and the supernatants were boiled for 5 min and filtered with syringe filter (0.2㎛ pore size) to be used for chemical analysis. Acetate, propionate, butyrate, and lactate concentrations were determined by 1260 Infinity HPLC (Aglient, CA, USA) on an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad). Degassed 5 mM H_2SO_4 was used as an eluent at a flow rate of 0.6 mL/min at 20 °C. SCFAs were detected by UV (Agilent, CA, USA) at a wavelength of 215 nm, and calibrated against standards of corresponding SCFAs at concentrations between 1 and 200 mM. The injection volume was 20 μL.

2.6. Statistics

Statistical analyses of the individual fermentation, quantitative real-time PCR (qPCR), and SCFAs were performed with the SPSS 22.0 software (SPSS Inc., USA), whereas principal component analysis (PCA) was carried out using the R software (version 3.4.2) to investigate the difference between the carbohydrate incubations and determine the bacterial taxa important for the fermentation of the tested compounds.

3. Results

3.1. Utilization of seaweed polysaccharides by individual bacteria

Laminaran, porphyran, or ulvan was added to culture media as the sole carbon source and fermentability by each of 23 individual microbial strains was analyzed after 48 h by changes of optical density. When

Table 3
Cell growth and pH changes in BHI medium containing laminaran, porphyran, and ulavan after anaerobic cultivation of individual gut microbiota for 48 h.

Individual gut microbiota	Laminaran		Porphyran		Ulvan	
	Difference in growth (ΔO.D)	Decrease in pH	Difference in growth (ΔO.D)	Decrease in pH	Difference in growth (ΔO.D)	Decrease in pH
Lactobacillus brevis ATCC 14869	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Lactobacillus acidophilus ATCC 4356	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Lactobacillus reuteri ATCC 23272	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Lactobacillus rhamnosus ATCC 53103	0.57 ± 0.14^{EF}	0.37 ± 0.04^{fgh}	0.32 ± 0.03^{GHI}	0.34 ± 0.03^{gh}	0^{J}	0^{j}
Lactobacillus plantarum ATCC 10241	0.24 ± 0.09^{HI}	0.34 ± 0.10^{gh}	0.30 ± 0.06^{HI}	0.33 ± 0.06^{gh}	0.37 ± 0.04^{GH}	0.34 ± 0.02^{fgh}
Weissella confusa KACC 11841	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Weissella cibaria KACC 11845	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Enterococuss faecalis ATCC 29212	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Bifidobacterium breve ATCC 15700	0.98 ± 0.08^{A}	0.76 ± 0.02^{a}	0^{J}	0^{j}	0.55 ± 0.01^{EF}	0.53 ± 0.03^{de}
Bifidobacterium longum ATCC 15707	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Bifidobacterium bifidum ATCC 29521	0^{J}	0^{j}	0.45 ± 0.04^{FG}	0.26 ± 0.04^{hi}	0^{J}	0^{j}
Bacteroides fragilis ATCC 25285	0.74 ± 0.02^{CD}	0.64 ± 0.02^{bc}	0^{J}	0^{j}	0.66 ± 0.04^{DE}	0.52 ± 0.04^{de}
Bacteroides vulgatus ATCC 8482	0^{J}	0^{j}	0^{J}	0^{j}	0.18 ± 0.04^{I}	0.20 ± 0.02^{i}
Bacteroides ovatus ATCC 8483	0.53 ± 0.10^{EF}	0.55 ± 0.09^{cd}	0.31 ± 0.02^{GHI}	0.32 ± 0.02^{h}	0.31 ± 0.03^{GHI}	0.30 ± 0.02^{hi}
Bacteroides dorei DSM 17855	0.93 ± 0.03^{AB}	0.73 ± 0.01^{ab}	0.82 ± 0.02^{BC}	0.43 ± 0.02^{efg}	0^{J}	0^{j}
Bacteroides thetaiotaomicron ATCC 29148	0^{J}	0^{j}	0^{J}	0^{j}	0.57 ± 0.02^{EF}	0.45 ± 0.03^{def}
Bacteroides uniformis ATCC 8492	0^{J}	0^{j}	0^{J}	0^{j}	0.33 ± 0.01^{GH}	0.29 ± 0.04^{hi}
Clostridium perfringens ATCC 13124	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Bacillus cereus ATCC 10876	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Escherichia coli ATCC 11775	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Listeria monocytogenes ATCC 19115	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Salmonella typhimurium ATCC 14028	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}
Staphylococcus aureus ATCC 6358	0^{J}	0^{j}	0^{J}	0^{j}	0^{J}	0^{j}

Different superscripted uppercase and lowercase letters indicate significant difference in growth and pH, respectively ($p < 0.05$).

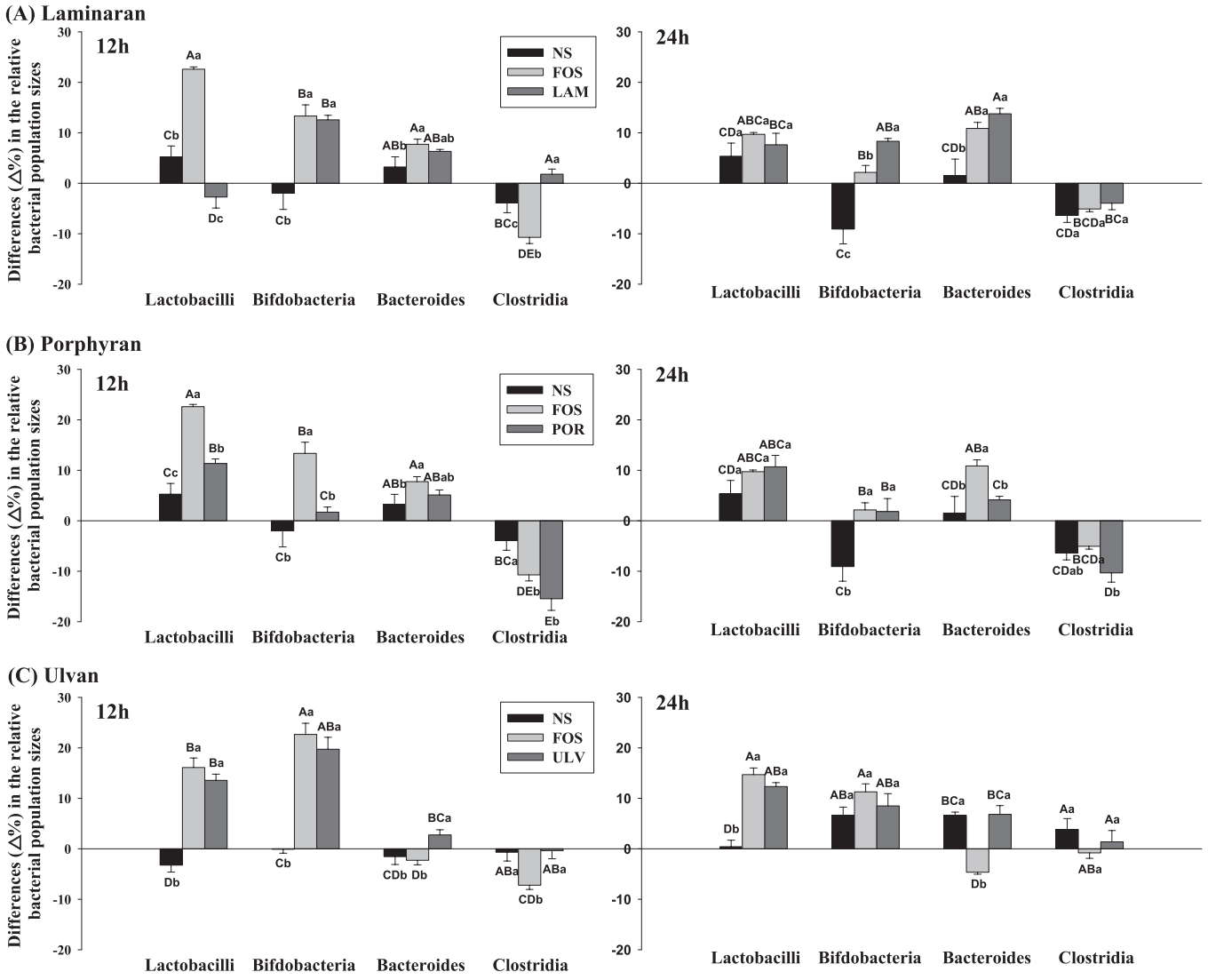

Fig. 1. Differences (Δ%) in the relative bacterial population sizes compared to the total number of bacteria counted at 12 and 24 h after fermenting laminaran (A), porphyran (B), and ulvan (C). The following calculation was used: [(selected bacterial number at 12 or 24 h/total bacteria number at 12 or 24 h) − (selected bacterial number at 0 h/total bacteria number at 0 h)] × 100. NS, no substrate; FOS, fructooligosaccharides; LAM, laminaran: POR, porphyran; ULV, ulvan. Error bars represent standard deviation of triplicates. Different letters indicate significant differences ($p < 0.05$). The capital letters indicate the differences among the tested seaweed polysaccharides for the population of same microbial genus, and the small letters indicate the differences among NS, FOS, and individual seaweed polysaccharides for the population of same microbial genus.

laminaran was added, *Lactobacillus rhamnosus, Lb. plantarum, Bifidobacterium breve, Bacteroides fragilis, Ba. ovatus,* and *Ba. dorei* exhibited a significant growth (OD_{580nm} 0.2–1.0) and pH reduction (0.3–0.7) (Table 3). In the presence of porphyran, *Lb. rhamnosus, Lb. plantarum, B. bifidum, Ba. ovatus,* and *Ba. dorei* exhibited significant growth (OD_{580nm} 0.3–0.8) and pH reduction (0.3–0.4). When ulvan was added, *Lb. plantarum, B. breve, Ba. fragilis, Ba. vulgatus, Ba. ovatus, Ba. thetaiotaomicron,* and *Ba. dorei* exhibited a significant growth (OD_{580nm} 0.2–0.7) and pH reduction (0.2–0.5). Meanwhile, the harmful or pathogenic bacteria (6 species) did not consume laminaran, porphyran, or ulvan, maintaining their cell densities at the basal levels ($OD_{580nm} < 0.2$).

3.2. Changes in fecal microbial communities after in vitro fermentation

In vitro fecal fermentation was conducted to confirm the effects of laminaran, porphyran, and ulvan on the growth of a mixed bacterial population in the colon. Fecal samples from 5 adults were mixed with 1% of laminaran, porphyran, or ulvan in basal media and incubated in an anaerobic, pH-controlled *in vitro* gut fermenter. For the comparison of prebiotic properties, FOS was also tested in a separate fermenter as a positive control. When the levels of different bacterial groups were quantified by genus-specific real time qPCR, the presence of laminaran, porphyran, or ulvan showed growth stimulating effects on all or some genera, such as *Lactobacillus*, *Bifidobacteria*, and *Bacteroides*, except *Clostridium*. As shown in Fig. 1A, in *in vitro* batch cultures supplemented with laminaran and FOS, a significant increase in the population of *Bifidobacteria* and *Bacteroides* was seen after 12 h and 24 h incubation, compared to the total number of bacteria. *Bifidobacterium* spp. increased 12.6% and 8.6% after 12 h and 24 h incubation, respectively, and *Bacteroides* spp. increased 6.3% and 13.8% after 12 h and 24 h incubation, respectively. This result is consistent with that of Table 3; exceptions include *Lb. rhamnosus* and *Lb. plantarum*. Notably, laminaran showed a slow bifidogenic effect for 24 h compared to FOS which was fast depleted at 24 h. As shown in Fig. 1B, when porphyran was used as the sole carbon source in the medium, *Bifidobacterium* spp. and *Bacteroides* spp. were slightly increased (1.7–5.7%) throughout incubation, while *Lactobacillus* spp. showed 11.3% and 10.7% increase compared to

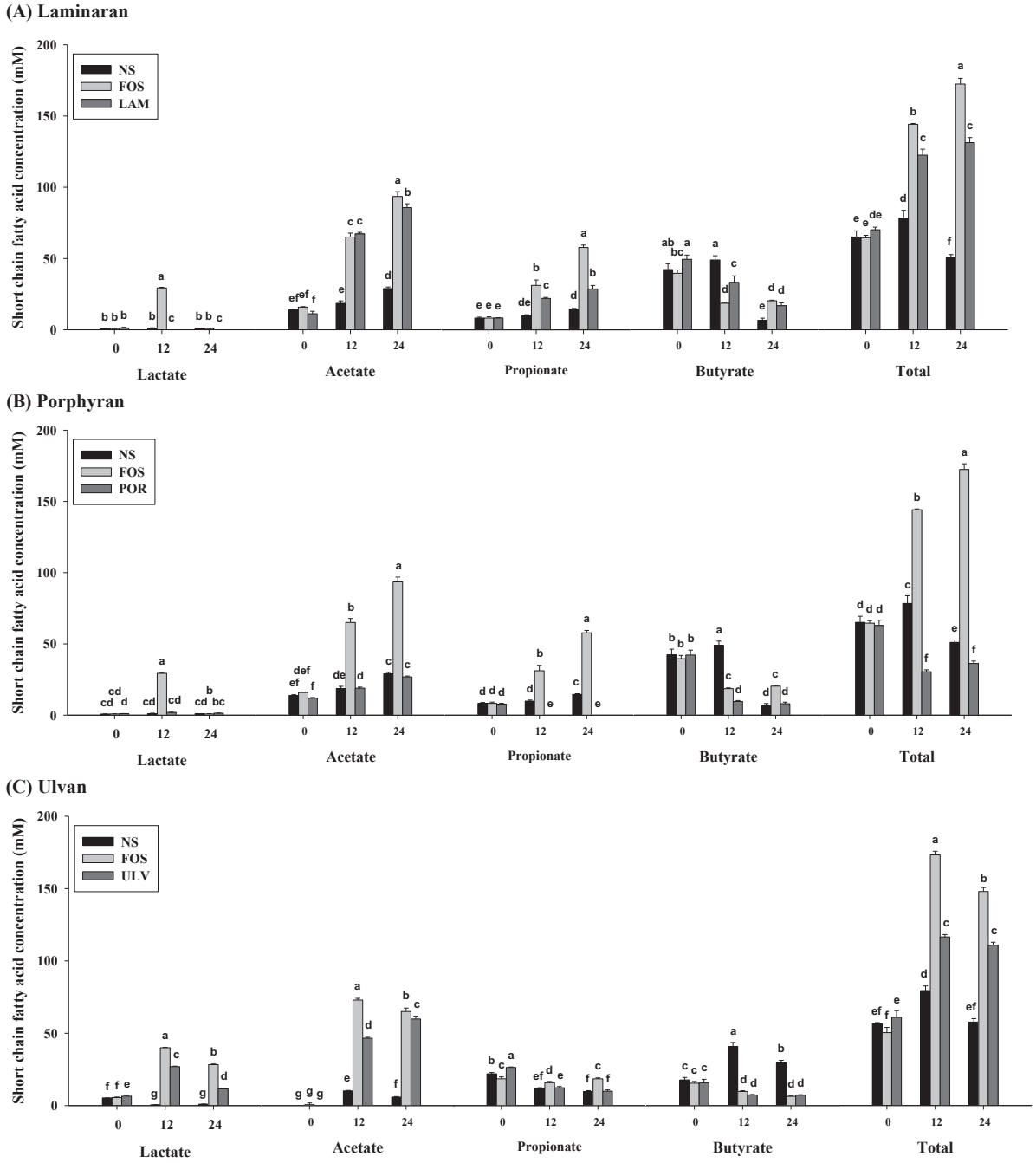

Fig. 2. Changes in lactate and short-chain fatty acid (SCFA) concentrations at 0, 12, and 24 h during *in vitro* fecal fermentation of laminaran (A), porphyran (B), and ulvan (C). NS (no substrate), FOS (fructooligosaccharides), LAM (laminaran), POR (porphyran), and ULV (ulvan). Error bars represent standard deviation of triplicates. Different letters indicate significant differences in concentrations of each short-chain fatty acid by addition of individual seaweed polysaccharides according to incubation times ($p < 0.05$).

total number of bacteria after 12 h and 24 h, respectively. This result is consistent with that of Table 3, except for *B. bifidum*. Notably, porphyran also showed a slow prebiotic effect on *Lactobacillus* spp. for 24 h compared to FOS. As shown in Fig. 1C, in the presence of ulvan, the population sizes of *Lactobacilli* and *Bifidobacteria*, compared to the total number of bacteria, were increased after 12 h and 24 h. *Lactobacillus* spp. showed 13.6% and 12.3% increase in population size after 12 h and 24 h, respectively, and *Bifidobacterium* spp. increased 19.7% and 8.5% in population size after 12 h and 24 h, respectively. Interestingly, in case of ulvan addition, the comparative population size of *Bacteroides* spp. slowly increased throughout the incubation from 2.8% increase at 12 h to 6.7% increase at 24 h. This result is consistent with that of Table 3; thus, ulvan stimulates the growth of the respective species belonging to *Lactobacilli, Bifidobacteria*, and *Bacteroides*. Like laminaran and porphyran, ulvan showed a slow prebiotic effect than FOS, stimulating the growth of commensal bacteria for 24 h.

3.3. SCFA production during in vitro fecal fermentation

It has been known that *Lactobacilli* and *Bifidobacteria* mainly synthesize lactate and acetate, respectively, as the final metabolites (El Aidy et al., 2013; Macfarlane & Macfarlane, 2003; Siebold et al., 1995). In case of *Bacteroides*, final metabolites are not distinctive; different SCFAs such as acetate, lactate, propionate, and butyrate can be produced depending

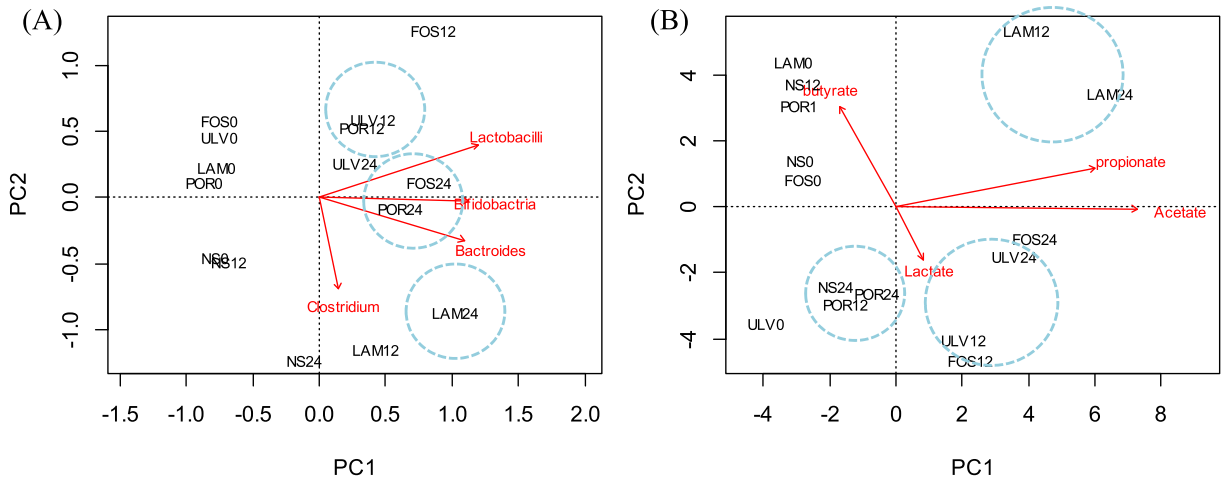

Fig. 3. Principal component analysis of cell growth and SCFAs. (A) Principal components analysis of cell growth using the first and second principal components (PC1, 26.80%; PC2, 5.08%). Loading plot indicating each of the bacterial taxa measured, as determined by quantitative real-time PCR. (B) Principal components analysis of SCFAs using the first and second principal components (PC1, 18.11%; PC2, 19.48%). Loading plot indicating each of the SCFAs measured, as determined by HPLC. Dotted circles indicate the same prebiotic effect of tested polysaccharides.

on growth phases (initial *vs.* late), medium composition (presence and absence of amino acids), and metabolic cross-feeding (consumption of lactate to produce acetate) (Adamberg et al., 2014).

In our study, when 1% each of laminaran, porphyran, or ulvan was fermented with human feces in basal media and the change in the concentration of SCFAs was analyzed using HPLC, the seaweed samples showed different production profiles depending on the polymers (Fig. 2). As shown in Fig. 2A, fermentation of laminaran resulted in high production of acetate and propionate showing concentrations of 85.7 mM and 28.7 mM after 24 h, respectively; similar production levels of total SCFAs were observed with FOS. This result is consistent with the data presented in Table 3 and Fig. 1, showing increased growth of *Bifidobacteria*. However, in the presence of porphyran, no significant changes in SCFAs were detected in the medium during the fermentation (Fig. 2B). This result might be due to less growth of the tested bacteria in the medium as shown in Fig. 1B. In case of ulvan, lactate (27.0 mM) and acetate (46.6 mM) were produced after 12 h, but acetate (59.9 mM) was mainly detected after 24 h despite lactate concentration decreased to 11.5 mM (Fig. 2C). This result revealed the typical cross-feeding effect among intestinal bacteria to produce acetate as the final metabolite from lactate as substrate (Adamberg et al., 2014).

3.4. Principal component analysis (PCA)

The prebiotic candidate groups were subjected to PCA to generate an overview of the fluctuation in the ability of the microbial communities to produce SCFAs from healthy subjects. Data were visualized in 2 dimensions using a principal component score (Fig. 3). The samples before (original bacterial communities) and after incubation of prebiotic candidates were clearly marked by PC1, with the fermented samples clustering to the right of the PCA plot. Fig. 3A shows that, during fecal fermentation, FOS positively influenced the populations of *Bifidobacteria* and *Lactobacilli*, while porphyran positively influenced the populations of *Lactobacilli* and *Bacteroides*. Meanwhile, NS (no substrate, control) negatively influenced the populations of *Clostridium*. As shown in Fig. 3B, FOS and ulvan increased the production of acetate and other SCFAs; however, NS decreased the production of those compounds.

4. Discussion

Laminaran is extracted from *Saccharina japonica* (formerly *Laminaria japonica*), which is brown seaweeds; porphyran is from *Pyropia* and *Porphyra*, a red algae; and ulvan is from *Chlorophyta,* a green seaweed, and they are most important seaweed polysaccharides in human food usage. In addition, they are used in pharmaceuticals and cosmetics owing to their different bioactivities. According to FAO statistics (FAO, 2014, 2016), these leading genera – *Saccharina*, *Undaria*, *Porphyra*, *Eucheuma/Kappaphycus*, and *Gracilaria* represent 98% of the world's cultivated seaweed production (Pereira & Yarish, 2008). In 2014, the leading seaweed farming countries were China, Indonesia, Philippines, and Korea; China and Indonesia each produced more than 10 million tons and Philippines and Korea produced over 1 million tons (FAO, 2014, 2016). Owing to the increasing evidence that the consumption of algal food products has health and nutritional benefits, application of those polysaccharides is supposed to increase in the future. Among their health benefits as a dietary fiber, prebiotic effects are regarded as one of the promising features that would influence on the microbiome and gut-related diseases.

The function of dietary fiber and prebiotics is to carry out fermentation in the large intestine, thus altering the intestinal microbial population (Gibson et al., 2010). Prebiotic compound must be resistant to digestion in the upper GI tract so that it can reach the lower intestine in an intact form (Roberfroid, 2008). This can be investigated *in vitro* by testing their resistance to acidic and enzymatic hydrolysis. Studies have shown that laminaran and porphyran remained intact during *in vitro* incubation with HCl, human saliva, and human gastric, pancreatic, small intestinal and colonic homogenates (Devillé, Damas, Forget, Dandrifosse, & Peulen, 2004; Muraoka et al., 2008; Neyrinck, Mouson, & Delzenne, 2007). In addition, ulvan was found to be resistant to both human digestive tract enzymes and degradation by colonic bacteria (O'Sullivan et al., 2010).

Studies conducted on seaweed extracts found that polysaccharides from brown seaweeds function as a good prebiotic that encourages the growth of beneficial bacteria in the intestines (Charoensiddhi et al., 2016; Kong, Dong, Gao, & Jiang, 2016). In our study using *in vitro* fecal fermentation, addition of laminaran, a brown seaweed polysaccharide, resulted in growth stimulation effect on *Bifidobacteria* and *Bacteroides* leading to the production of acetate and propionic acid. Our result is consistent with previous reports; Lynch, Sweeney, Callan, O'Sullivan, and O'Doherty (2010) reported that *Bifidobacteria* can selectively use laminaran, and it was reported that *Bifidobacteria* produce acetate and *Bacteroides* produce acetate and propionate (Macfarlane & Macfarlane, 2003).

In our study, laminaran, ulvan, and FOS were fermented by *Bifidobacteria*, but laminaran was fermented more slowly than ulvan or FOS. It has been reported that quick fermentation of carbon sources can

Table 4
Presumed bacterial enzymes (genes) involved in utilization of seaweed polysaccharides.

	Seaweed hydrolysis enzyme	*Lb. reuteri* ATCC 23272	*Lb. rhamnosus* ATCC 53103	*Lb. plantarum* ATCC 10241	*B. bifidum* ATCC 29521
Laminaran	Laminarinase (EC 3.2.1.39)	–	–	–	–
	Periplasmic β-glucosidase (EC. 3.2.1.21)	–	–	–	–
	β-glucosidase (EC. 3.2.1.21)	–	GCF_000011045.1_00097	–	–
	Glucan 1, 3-beta-glucosidase (EC. 3.2.1.58)	–		–	–
Porphyran	6-phospho-β-glucosidase (EC. 3.2.1.86B)		GCF_000011045.1_00166		
	β-galactosidase (EC. 3.2.1.23)			GCF_00014374531_02690	GCF_001025135.1_00010
				GCF_00014374531_02703	GCF_001025135.1_00070
					GCF_001025135.1_00497
					GCF_001025135.1_01325
					GCF_00102513
Ulvan	β-glucuronidase (EC. 3.2.1.31)		GCF_000011045.1_00051		
	α-L-rhamnosidase (EC. 3.2.1.40)			GCA_000143745.1_02692	
				GCA_000143745.1_02694	
	Glucan 1,4- α-glucosidase SusB (EC. 3.2.1.20)				
	β-glucosidase (EC. 3.2.1.21)				

	B. breve ATCC 15700	*Ba. fragillis* ATCC 25285	*Ba. ovatus* ATCC 8483	*Ba.vulgatus* ATCC 8482	*Ba. thetaiotamicron* ATCC 29148	*Ba. dorei* DSM 17855
Laminaran	–	–	–	–	–	GCF_000156075.1_00611
	–	–	–	–	–	GCF_000156075.1_02640
	GCF_001025171.1_00125	GCF_000025985.1_00269	GCF_001314995.1_01323	–	–	GCF_000156075.1_02656
	GCF_001025171.1_01973		GCF_001314995.1_01538			GCF_000156075.1_03740
	GCF_001025171.1_01521	–	–	–	–	
Porphyran						
			GCF_1314995.1_00033			GCF_000156075.1_00391
			GCF_1314995.1_00172			GCF_000156075.1_01073
			GCF_1314995.1_00595			GCF_000156075.1_01082
Ulvan						
		GCA_000025985.1_00304	GCA_001314995.1_00597	GCA_000012825.1_00182	GCA_000011065.1_01053	GCA_000156075.1_01032
		GCA_000025985.1_00525	GCA_001314995.1_00601	GCA_000012825.1_01860	GCA_000011065.1_01064	GCA_000156075.1_01035
			GCA_001314995.1_00642	GCA_000012825.1_02726	GCA_000011065.1_01070	GCA_000156075.1_01172
				GCA_000012825.1_00140	GCA_000011065.1_00709	
				GCA_000012825.1_00650	GCA_000011065.1_03309	
				GCA_000012825.1_01043		
				GCA_000012825.1_00138	GCA_000011065.1_01859	
				GCA_000012825.1_00585		

cause initial production of gases in the human large intestine that can result in discomfort, such as bloating and flatulence (Halmos et al., 2015; Staudacher, Irving, Lomer, & Whelan, 2014). Therefore, to minimize these symptoms, slow fermentation would be desirable. In addition, slow fermentation will permit production of SCFAs throughout the colon, and this may be effective in preventing colon cancer, which mainly occurs in the distal colon (Koh et al., 2016; Monteagudo-Mera et al., 2018; Ríos-Covián et al., 2016). Given the observed rate of fermentation, the laminaran exerting slow fermentation along with 24 h can be regarded as a favorable prebiotic substrate. In these experiments using laminaran, porphyran, and ulvan, acetate was the most abundant SCFAs detected in the medium and this result is consistent with the report that acetate was more than half of the total SCFAs detected in human feces (Louis, Scott, Duncan, & Flint, 2007). Three main metabolic pathways can be proposed for acetate production by the gut microbiota: by most enteric bacteria as a result of sugar fermentation, by acetogenic bacteria to synthesize from hydrogen and carbon dioxide or formic acid through the Wood–Ljungdahl pathway (Miller & Wolin, 1996), and by metabolite cross-feeding (Ríos-Covián et al., 2016).

To utilize seaweed polysaccharides as carbon sources, the intestinal microbiota should have proper hydrolysis enzymes or carbohydrate active enzymes (CAZymes), which are absent in the human genome (Cantarel et al., 2008). Considering the structure of laminaran having linear β-(1-3)-linked glucan with some random β-(1-6)-linked side-chains, we assumed that some enzymes, such as laminarinase, β-glucosidase, or glucan 1, 3-β-glucosidase are necessary to hydrolyze it. Therefore, we analyzed the presence of the corresponding genes in the genome of *B. breve* ATCC 15700 by using Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp) (Table 4). We found the existence of corresponding genes for the following enzymes: β-glucosidase (EC. 3.2.1.21), GCF_001025171.1_00125 and GCF_001025171.1_01973; glucan 1,3-β-glucosidase (EC. 3.2.1.58), GCF_001025171.1_01521. Fukuda, Imura, Kasahara, Kirimura, and Usami (1987) also found enhanced activities of β-glucosidase and β-l,3-glucanase during cultivation of microorganisms in laminaran containing medium. In case of porphyran having relatively complex backbone of 3-linked β-D-galactosyl units alternating with either 4-linked α-L-galactosyl 6-sulfate or 3,6-anhydro-α-L-galactosyl units, it was consumed by *Ba. dorei* DSM 17855 showing growth among tested bacteria (Table 4). When the presence of the corresponding genes was investigated in the genome, we found 3 genes (GCF_000156075.1_00391, GCF_000156075.1_01073, GCF_000156075.1_01082) corresponding to β-galactosidase (EC. 3.2.1.23), which showed hydrolytic activity towards porphyran in case of marine bacterium (Sunairi et al., 1995). Nevertheless, further studies are necessary to characterize the enzymes responsible for hydrolytic reactions of the complex backbone structure. In case of ulvan, composed of sulfated L-rhamnose, D-glucuronic acid, and its C5-epimer L-iduronic acid, and a minor fraction of D-xylose, it was utilized by *Ba. thetaiotaomicron* ATCC 29148 showing higher optical density among the tested bacteria. In the whole genome sequence of the bacterium, as possible enzymes involved in the hydrolysis, we found 3 genes (GCA_000011065.1_01053, GCA_000011065.1_01064, and GCA_000011065.1_01070) for α-L-rhamnosidase (EC. 3.2.1.40), 2 genes (GCA_000011065.1_00709 and GCA_000011065.1_03309) for glucan 1,4-α-glucosidase (EC. 3.2.1.20), and a gene (GCA_000011065.1_01859) for β-glucosidase (EC. 3.2.1.21). Moreover, *Ba. thetaiotaomicron* is known to be involved in the metabolism of various polysaccharides, because it has a diverse array of CAZymes (Xu et al., 2003): 261 glycoside hydrolases and polysaccharide lyases (Martens, Koropatkin, Smith, & Gordon, 2009).

5. Conclusions

We investigated the prebiotic effects of the three representative seaweed polysaccharides, having different monosaccharide compositions and structures, by using individual fermentability test and *in vitro* fecal fermentation. In individual culture test, laminaran, porphyran, and ulvan were selectively utilized by species of *Bifidobacteria, Lactobacilli, and Bacteroides*, while no growth of harmful bacteria was observed. *In vitro* fecal fermentation for 24 h also showed growth stimulation effect of the three polysaccharides on *Bifidobacteria, Lactobacillus, or Bacteroides*, leading to the production of short chain fatty acids. However, porphyran itself showed little prebiotic effect compared to laminaran and ulvan. This *in vitro* study demonstrated that the three seaweeds tested can mediate beneficial effects on human colon microbiota, and that particularly laminaran and ulvan exhibit better prebiotic effect than porphyran.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this study.

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