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Chemical structure and molecular weight influence the *in vitro* fermentability of polysaccharide extracts from the edible seaweeds *Himathalia elongata* and *Gigartina pistillata*

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ABSTRACT

Polysaccharides from the brown seaweed *Himanthalia elongata* and the red one *Gigartina pistillata* have been extracted by sequential fractionation based on the solubility in water (F-H_2O), hydrochloride acid (F-HCl) and potassium hydroxide (F-KOH), remaining a residue (RES). Their structures have been studied through Fourier-transform infrared spectroscopy (FTIR) and molecular weight by molecular exclusion (HPSEC). Sugar composition and sulphate content were also determined. F-H_2O and F-HCl from *Himanthalia elongata* were rich in fucoidans with an estimated Mw of 926 and 430 × 10^3 g/mol. Laminarans appeared in the mentioned fractions and possibly xylofucoglycuronans and xylomannans are in F-H_2O. F-HCl was poorly fermented with low production of total short chain fatty acids (SCFA) and two poor or non-fermentable low-molecular weight polyuronans (68 and 4 × 10^3 g/mol) appeared in F-KOH. Carrageenans extracted from *Gigartina pistillata* presented low or non-fermentability. Xylofucoglycuronans and xylomannans, possibly presented in RES and F-H_2O from *Himathalia*, and laminarans in the last mentioned fraction, seem to be more fermentable. Carragenophytes red seaweeds do not seem to be fermentable whereas brown algae could have major potential fermentability, probably due to the presence of laminarans, xylofucoglycuronans and/or xylomannans.

1. Introduction

Seaweeds are a common ingredient in Asian countries, however in western countries they are used mainly as thickening and gelling agents. Seaweeds are promising organisms for providing novel biologically-active ingredients, with a high impact on the food and pharmaceutical industry and public health (Gupta & Abu-Ghannam, 2011; Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2013a; LØvstad Holdt & Kraan, 2012; Roohinejad et al., 2017). Moreover, the edible seaweeds represent new sources of dietary fibre and can be utilized for beneficial purposes, since these bioresources are often regarded as underexploited (Gómez-Ordoñez, Jiménez-Escrig & Rupérez, 2010).

Himathalia elongata and *Gigartina pistillata* from Spanish coasts are edible seaweeds with high content of dietary fibre (DF, 29–37 g/100 g dry weight, d.m.), minerals (35–37 g/100 g d.m.) and protein (14–16 g/100 g d.m.), and they have a low lipid content (0.6–0.9 g/100 g d.m.) (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010). The fibre content is higher than those found in many fruits and vegetables. The brown seaweeds, i.e. *Himanthalia elongata*, are rich in different polysaccharides like fucans, laminaran, alginates and cellulose. Fucans, soluble in water and acid solution, can be classified into three groups: 1) fucoidans, (1 → 2)- and/or (1 → 3)-linked 4-O-sulphated fucopyronose,; 2) xylofucoglycuronans, poly-β-(1,4)-D-mannuronic acid branched with 3-O-D-xylosyl-L-fucose-4-sulphate or occasionally uronic acids; and 3) glycuronogalactofucans, linear chains of (1,4)-D-galactose branched with L-fucose-3-sulphate or occasionally uronic acids. Laminaran is water soluble (1,3)-β-D-glucans with β(1,6) branching. Alginates are generally alkali-soluble 1,4-linked-β-D-mannuronic and α-L-guluronic (Gupta & Abu-Ghannam, 2011). Carragenophytes red seaweeds, such as *Gigartina pistillata*, present high molecular weight sulphated carragenans (D-galactans), being the most important types kappa, iota and lamdda (O'Sullivan et al., 2010).

The physico-chemical properties reveal that the dietary fibre polysaccharides from these seaweeds could contribute to water binding, faecal bulking and decreased transit time, thus representing a good source of food fibre for human consumption (Gómez-Ordóñez et al., 2010). Being rich in polysaccharides, which are not digested, makes seaweeds important sources of dietary fibres with potential prebiotic

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effects. In fact, various *in vitro* and *in vivo* studies have shown the potential prebiotic effect of dietary fibre polysaccharides in brown seaweeds (Jiménez-Escrig, Gómez-Ordoñez, Tenorio, & Rupérez, 2013b; Villanueva et al., 2014). However, Dierick, Ovyn, and De Smet (2009) suggest low or no fermentability of brown algae polysaccharides by gut microbiota. The low molecular weight extracts from different species of agarphytes red seaweeds have been fermented by gut microbiota (Ramnani et al., 2012). Gómez-Ordoñez, Jiménez-Escrig, and Rupérez (2012a) and Villanueva et al. (2014) observed no clear prebiotic effect from dietary supplementation to Wistar rats with the carragenophytes red seaweeds *Mastocarpus stellatus* and *Gigartina pistillata*, respectively. The dietary fibres from seaweeds are physico-chemically different from those of terrestrial plants and may induce different fermentative patterns. Therefore, more research is necessary to elucidate the effects of seaweeds over the intestinal microbiota and gut health related with the different types of polysaccharides that constitute the cell wall. While the prebiotic activity must be determined *in vivo*, the *in vitro* studies are useful for preliminary screening of candidates and can generate information about the fermentability mechanism (Corzo et al., 2015).

The present study was carried out trying to evaluate the relation between the composition, the molecular weight and the chemical structures with the ability for *in vitro* fermentation of the polysaccharide-rich fractions extracted from both seaweeds, the brown *Himanthalia elongata,* and the red *Gigartina pistillata.*

2. Material and methods

2.1. Seaweed samples

The brown seaweed (Ochrophyta, Phaeophyceae) *Himanthalia elongata* (L.) S.F. Gray (sea spaghetti; Fucales, Himanthaliaceae), and the red seaweed (Rhodophyta) *Gigartina pistillata* (S.G. Gmelin) Stackhouse (Gigartinales, Gigartinaceae) from the Spanish Atlantic coasts were obtained from a local supplier (Porto-Muiños, A Coruña, Spain). At the industry, the seaweeds were washed with running water under the tap to remove epiphytes and sand, air-dried at 50 °C and milled to a particle size of less than 1.0 mm. The milled samples were stored sealed plastic bags at 4 °C until analysis.

2.2. Sequential extraction of seaweed polysaccharides

The seaweeds were defatted and depigmented in a Soxhlet system by extraction with diethyl ether solvent. These samples were extracted sequentially based on the method described by Rupérez, Ahrazem, and Leal (2002) with some modifications. Firstly, a water extraction in constant stir at 20 °C during 2h was carried out. Temperature was increased until 60 °C and kept during 17h (F-H_2O). The residue, after centrifugation at 9000 rpm, 10 min, was extracted with HCl 0.1 M at 37 °C during 6h (F-HCl). After collecting supernatant from centrifugation (9000 rpm, 10 min), the final extraction was carried out with KOH2M at 37 °C during 6h (F-KOH). Three fractions were collected and a residue remained. F-HCl and F-KOH were neutralized (pH 5.5 ± 0.1) with 2M KOH and HCl 37%, respectively. The residue was washed with 2M HCl and water until neutral pH. Each extract and the residue were dialyzed with 12–14 KDa membranes (Medicell Internacional Ltd.) against tap water at room temperature during at least 24 h. Conductivity was measured using a conductivity meter to ensure the salt elimination. Each fraction was then freeze-dried.

2.3. Analysis of seaweed polysaccharides fractions

2.3.1. Neutral sugar composition, uronic acid and sulphate contents

All the fractions were studied for their neutral sugars by gas chromatography according to Englyst, Quigley, and Hudson (1994), using β-D-allose (Fluka) as internal standard. The samples were treated with H_2SO_4 12M at 35 °C during 30 min, followed by H_2SO_4 2M at 100 °C during 1 h. The released neutral sugars were transformed into alditol acetates with acetic anhydride in the presence of 1-methylimidazol. Quantification was performed in a Perkin-Elmer Autosystem Chromatograph equipped with a hydrogen flame ionization detector. The column used was a SP-2330 (30 m long, 0.25 mm i.d., and 0.25 μm film thickness) and nitrogen served as carrier gas. Temperatures of injector and detector were 275 °C and oven temperature was 235 °C.

Uronic acid content was determined in the acid hydrolysates according to the colorimetric method of 3,5-dimethylphenol (Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Ruperez, 2010), with a UV/Vis Perkin–Elmer Lambda EZ210 spectrophotometer, using galacturonic acid (Merck) as standard (20–120 μg/mL).The calibration curve was y = 0.0107x - 0.1206 (r^2 = 0.999).

The sulphate determination was carried out by the gelatin-barium chloride turbidimetric method (Rupérez et al., 2002). The extracts were mixed with trichloroacetic acid reagent and gelatin-barium chloride reagent. Absorbance was measured at 500 nm in an UV/Vis Perkin–Elmer Lambda EZ210 spectrophotometer. Sulphate content in the samples was calculated with a calibration curve of K_2SO_4 in distilled water (0.25–1 mg/mL). The calibration curve was y = 0.2461x + 0.0537 (r^2 = 0.989).

2.3.2. Fourier transform infrared (FTIR) analysis

The polysaccharide fractions from both seaweeds and the standards fucoidan, laminarin, alginate, Lambda-carragenan and Kappa-carragenan were incorporated into KBr and pressed into a 1 mm disk. Fucoidan (F8190), Laminarin (L9634), Alginate (alginic acid sodium salt, W201502), Lambda- (22049) and Kappa-carrageenans (22048) were obtained from Sigma-Aldrich. FTIR spectra of samples were recorded in the transmittance mode from 4000 to 400 cm^{-1} using a FT-IR Nicolet Magna 750 spectrometer (Mateos-Aparicio et al., 2010; Gómez-Ordóñez & Rupérez, 2011).

2.3.3. Mw determination

The molecular weight determination was carried out in seaweed polysaccharides fractions through HPSEC analysis following the method described by Gómez-Ordoñez, Jiménez-Escrig, & Rupérez (2012b). The HPLC system was equipped with the following instruments: Autosampler Agilent 1200 Series, quaternary pump system, thermostatic oven, differential refractometer (RI detector) (Agilent 1100, Santa Clara, CA, USA) and the software HPCHEM Agilent. The separation was performed on a Polysep-GFC-P Linear (300 × 7.8 mm) with a Polysep-GFC-P guard column (35 × 7.8 mm) from Phenomenex (Micron-Analítica, Madrid, Spain). The column was eluted isocratically with 0.1 M sodium nitrate at 40 °C with a flow rate of 0.8 mL/min.

A Shodex pullulan standard P-82 kit (range of molecular weights in kDa: P-800 = 788, P-400 = 404, P-200 = 212, P-100 = 112, P-50 = 47.3, P-20 = 22.8, P-10 = 11.8, P-5 = 5.9; Showa-Denko, Japan) was obtained from Waters (Madrid, Spain). Each of these standards was separately injected at 0.5, 1.0 and 2.0 mg/mL (in ultrapure water solution) to perform the calibration curves. Standards and polysaccharide fractions were filtered through 0.45 μm cellulose acetate filters (Micron-Analítica, Madrid, Spain) and injected (50 μL) into the HPLC. Under the conditions used chromatographic runs took less than 15 min.

2.4. In vitro fermentation of seaweed polysaccharides fractions

Fermentations were performed using an *in vitro* batch culture system under strict anaerobic conditions based on the method describe previously by Goñi, Gudiel-Urbano, Bravo, and Saura-Calixto (2001). One hundred milligrams of each substrate, lactulose (L-7877 from Sigma-Aldrich Química S.A. Madrid, Spain, used as positive control) and studied samples, were weighed into 50 mL serum vials and 8 mL of fermentation medium were added into each vial. The medium contained trypticase, micromineral, macromineral solutions and resazurin as anaerobic redox indicator. Vials were sealed with rubber caps (no.

Table 1
Monomeric composition (g/100 g dry weight) of polysaccharides fractions from *Himathalia elongata*.

Fraction	Yield	Neutral sugars							UA	Total	Sulphate
		Rha	Fuc	Ara	Xyl	Man	Gal	Gluc			
F-H_2O	26.3	–	17.9 ± 1.6^{d}	0.2 ± 0.0^{b}	0.9 ± 0.2^{c}	2.4 ± 0.3^{c}	4.5 ± 0.4^{c}	17.5 ± 1.8^{b}	19.6 ± 1.3^{c}	63	7.0 ± 0.4^{b}
F-HCl	2.2	–	8.2 ± 1.5^{c}	0.3 ± 0.1^{b}	0.5 ± 0.1^{b}	1.4 ± 0.2^{b}	13.8 ± 1.8^{d}	24.1 ± 3.4^{c}	2.9 ± 0.5^{b}	51.2	6.4 ± 0.2^{b}
F-KOH	29.3	–	2.2 ± 0.2^{b}	0.1 ± 0.0^{a}	0.3 ± 0.0^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{b}	3.2 ± 0.2^{a}	27.8 ± 1.4^{d}	34.6	1.1 ± 0.4^{a}
RES	6.3	–	0.7 ± 0.0^{a}	–	0.9 ± 0.1^{c}	3.4 ± 0.2^{d}	0.3 ± 0.1^{a}	89.8 ± 6.2^{d}	0.2 ± 0.1^{a}	95.3	–

Yield (%) by gravimetry (g/100 g dry weight).Total is the sum of neutral sugars and uronic acids.
Different letters indicate significant ($p < 0.05$) differences between fractions.

407-0-13, Ormacisa, Madrid, Spain), and substrates were left overnight at 4 °C to hydrate. Two millilitres of inoculum was added into each vial and the headspace rinsed with carbon dioxide for 1 min. The inoculum was obtained from the caecal contents of rats killed in a carbon dioxide chamber. Those caecal contents were collected and introduced into a flask containing sterilized anaerobic medium to give a final 100 g L^{-1} (10% w/v). The inoculum was mixed in a Stomacher 80 Lab Blender (Seward Medical, London, U.K.) before use.

Vials were placed in a shaking water bath at 37 °C for 24 h. Fermentations were prolonged during 24 h using oxygen free carbon dioxide to maintain anaerobiosis. Substrates and blanks (inoculum without substrate) were fermented in triplicate. After 24 h, the pH and gas pressure was measured in each vial. Fermentations were then stopped by adding an excess of 1 M sodium hydroxide (2.5 mL). Samples were centrifuged at 2500g for 10 min and supernatants (3 mL) separated in duplicate for short chain fatty acid (SCFA) determination.

2.5. SCFA analysis

The supernatants separated by centrifugation were utilized for gas-liquid chromatography using 4-methyl valeric acid as internal standard (Jiménez-Escrig, Tenorio, Espinosa-Martos, & Rupérez, 2008). 1 μl of supernatant was injected into a GLC chromatograph (Perkin-Elmer Autosystem, Waltham, MA) equipped with a flame ionization detector and a fused silica column (Carbowax 20 M, 10 m × 0.53 mm × 1.33 μm film thickness). The carrier gas was nitrogen with a flow rate of 15 mL/min. The temperature for the injector and detector was 250 °C and the column temperature was isothermal at 120 °C.

The percentage of fermentability was calculated as the relative percentage of total SCFA produced by the polysaccharides fractions with respect to the total SCFA produced by the lactulose within 24 h.

2.6. Statistical analysis

Statistical analysis was performed using Statgraphics Centurion XVI (Warrenton, VA, USA). Significant changes were analysed by one-way analysis of variance (ANOVA, $p < 0.05$) and later post-hoc multiple range analyses by using a Duncan test.

3. Results and discussion

3.1. Analysis of polysaccharide fractions from the seaweeds Himanthalia elongata

The procedure of extraction separates different fractions depending on the different solubility of the polysaccharides. The M_W and the sugar composition were studied in the different fractions obtained and in the remaining residue (F-H_2O, F-HCl, F-KOH and RES). Moreover, the soluble fractions were studied by FTIR analysis and compared with different polysaccharide standards (alginate salt, fucoidan and laminaran), and also to the whole seaweed *Himathalia*.

3.1.1. Determination of the monosaccharide composition

Table 1 shows the monomeric composition of the extracted fractions from *Himathalia elongata*. The yields showed that the most important fractions are the water-soluble and alkali-soluble ones. The composition of these fractions is very different, with fucose being the most important sugar in F-H_2O, and, however, the content of uronic acids was the greatest in F-KOH. Moreover, the highest content of sulphate was in F-H_2O and the lowest in F-KOH, highlighting the presence of fucoidans, (1 → 2)- and/or (1 → 3)-linked 4-O-sulphated fucopyronose, in the water-soluble fraction, and alginates, 1,4-linked-β-D-mannuronic and α-L-guluronic, in the alkali one (Gupta & Abu-Ghannam, 2011; Rupérez et al., 2002). The notable amount of uronic acids in F-H_2O could be due to water-soluble alginates (Lee & Mooney, 2012). The remarkable content of glucose in F-H_2O and F-HCl indicate the presence of laminarans, (1,3)-β-D-glucans, and the higher content of glucose in the hydrochloric acid fraction points to a major extraction of these polysaccharides in mild acid conditions. The high amount of uronic acid, fucose and sulphate, together with the presence of xylose in F-H_2O could indicate the presence of xylofucoglycuronans, which consist on a polyuronide backbone branched with 3-O-D-xylosyl-L-fucose-4-sulphate or occasionally uronic acids, described previously in *Himanthalia* genus (Praiboon, Chirapart, & Sisarp, 2016). Moreover, the presence of minor amounts of xylomannans could be possible. This polysaccharide is not common in brown seaweeds, although it was described in *Dictyota dichotoma* (Rabanal, Ponce, Navarro, Gómez, & Stortz, 2014) and *Sphacelaria indica* (Vavilala & D'Souza, 2015), both brown seaweeds. F-HCl presented a notable content of galactose, fucose and remarkable amount of sulphate. This composition might be related with glycuronogalactofucans, which are linear chains of (1,4)-D-galactose branched at C5 with L-fucose-3-sulphate or occasionally uronic acid (Gupta & Abu-Ghannam, 2011). However, this type of fucan has not been described in the *Himanthalia* genus before. The rich in glucose residue was composed by cellulose and probably minor amounts of non-extractable glycuronogalactofucans and/or xylomannans.

3.1.2. Structural analysis with fourier-transform infrared spectroscopy (FTIR)

FTIR region between 4000 and 2000 cm^{-1} showed two important bands, one broad band around 3400 cm^{-1} assigned to hydrogen bonded O-H stretching vibrations, and another around 2900 cm^{-1}, more intense for the standard Laminaran, the seaweed *Himanthalia* and the F-HCl, due to C-H stretching vibrations (Gómez-Ordóñez & Rupérez, 2011).

FTIR-ATR spectra in the range of 2000-650 cm^{-1} are represented in the Fig. 1. This region presents ten remarkable bands. The first one is observed at 1730 cm^{-1}, but this just appear in the Fucoidan standard related with carboxylic ester form (C=O). The second band at 1600 cm^{-1}, corresponding with carboxylate anion form (COO^-), appears in Alginate standard and in F-H_2O and F-HCl extracts. This band in F-H_2O, together with the great amount of uronic acids (Table 1), could indicate the presence of water-soluble alginates. The other fractions present a band at 1620-1640 cm^{-1} that might be related also with the carboxylate anion. The band 3 at 1420 cm^{-1} appears in all samples

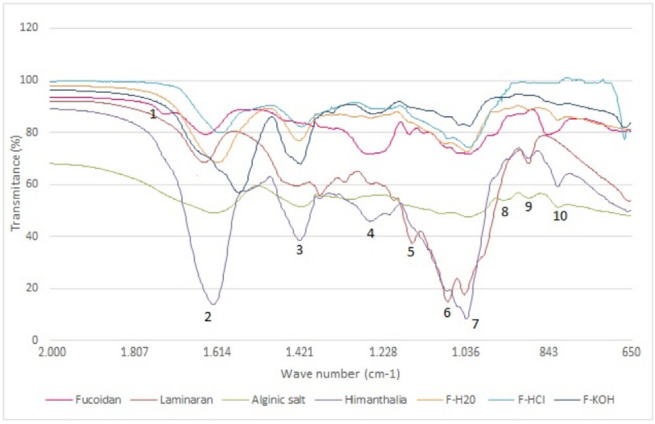

Fig. 1. Infrared spectra of polysaccharide standards: fucoidan, laminaran and alginic salt and *Himanthalia elongata* fractions: whole *Himanthalia elongata*, water-extracted fraction (F-H20), hydrochloride-extracted fraction (F-HCl) and potassium hydroxide-extracted fraction (F-KOH) Band numbers (1–10) in FTIR spectra indicate most characteristic bands.

except in the standards Fucoidan and Laminaran, and it is related with the presence of uronic acids (Table 1) as was previously described Rupérez et al. (2002). All soluble fractions presented uronic acids, although it was scarce in F-HCl (Table 1), and it was supported by the lowest intensity at 1420 cm^{-1} of this fraction. The broad band 4 between 1220 and 1255 cm^{-1} was due to sulphate ester groups (S = O) and it was observed very intense in Fucoidan standard and *Himathalia* and less intense in the extracted fractions. Laminaran standard did not present the described band. Band 5 at 1150-1160 cm^{-1} for –C–O–C– groups vibration in glycosidic linkages is identified in all analyzed samples although the intensity was major in Laminaran standard and *Himanthalia*. Between 1080 and 1020 cm^{-1} two bands were observed (Fig. 1, Bands 6,7) related with C-O and C-C stretching vibrations of pyranose ring (Gómez-Ordóñez & Rupérez, 2011) which appears clearly in Laminaran standard and *Himathalia*. These two bands showed, however, very little intensity in the rest of the samples studied. The band 8 at 941 cm^{-1} is assigned to the C-O stretching vibration of uronic acid residues. At 881 cm^{-1} (Fig. 1, Band 9) the absorption band is due to C1-H deformation vibration of β-mannuronic acid residues. The band 10 at 816 cm^{-1} is characteristic of mannuronic acid. The three band (8–10) in the region of 950-800 cm^{-1} appeared in Alginate salt standard and *Himathalia*. K-OH fraction presented the absorption bands at 940 and 816 cm^{-1} (Fig. 1, Band 8, 10), indicating the presence of uronic acid residues and especially mannuronic acid in this extract. This is supported by the remarkable uronic acid content determined (Table 1). At 820 cm^{-1} (Band 10) a band appeared in F-H_2O that could be related with the presence of mannuronic acid residues. This is because of the water-soluble alginates and/or the polyuronide backbone, mainly poly-β-(1,4)-D-mannuronic from xylofucoglycuronans supported by the significant uronic acids amount determined in this fraction (Table 1).

3.1.3. Molecular weight distribution

The molecular weights (M_W) of the extracted polysaccharides fractions from *Himathalia* were studied (Table 2). F-H_2O and F-HCl presented just one peak, but two peaks were observed in F-KOH. The peaks of the different fractions have different and lower M_W through the process of sequential extraction. This means that the degree of polymerization became lower, giving as a result lower molecular weight distributions. A previous work about the brown seaweed *Saccharina latissima* (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2012b) describes peaks at retention time (RT) of 8 min in water and potassium hydroxide fractions with a large M_W (2428–2111 × 10^3 g/mol). We did not detect any peak at that time. These authors also detects in water fractions peaks at RT = 10.4 min with a M_W between 433–310 × 10^3 g/mol. The peak detected in our water (RT = 9.8) and hydrochloric (RT = 10.2) fractions presented high M_W (Table 2). The calculated M_W could be related to the fucoidans extracted in water and HCl fractions (Table 1; Fig. 1), with M_W reported between 100–1600 × 10^3 g/mol (Gupta & Abu-Ghannam, 2011; Rupérez et al., 2002) and also with water-soluble alginates (32–400 × 10^3 g/mol) (Lee & Mooney, 2012) in the case of F-H_2O. Laminarans usually have a M_W of 3–6 x 10^3 g/mol although it depends on polymerization degree (Gupta & Abu-Ghannam, 2011; Shevchenko et al., 2007). Menshova et al. (2014) described a high molecular weight laminaran (19–27 × 10^3 g/mol) from brown alga *Eisenia bicyclis*. The information about the isolation of high molecular weight laminaran from other brown algae is absent. These authors proposed that brown alga *E. bicyclis* produces different types of laminarans depending on the habitat and the harvesting time of alga. Laminarans with Mw lower than 12–14 × 10^3 g/mol are missing in dialysis process. However, the remarkable glucose content (Table 1) is indicating the presence of a laminaran with a molecular weight higher than 12–14 × 10^3 g/mol in F-H_2O and F-HCl, as Menshova et al. (2014) described in *Eisenia bicyclis*. Nevertheless, just one peak corresponding to high Mw associated to the presence of fucoidans and/or water-soluble alginates was identified in those fractions where laminarans were extracted (Table 1; Fig. 1), probably because of the overlapping with the observed broad and intense peak. The two peaks observed in F-KOH present minor M_W (68 and 4 × 10^3 g/mol) than in the previous fractions, and may correspond to alginates, which are the main alkali-soluble polysaccharides in brown seaweeds (Gómez-Ordóñez et al., 2012b; Rupérez et al., 2002). On the other hand, the alginates usually have greater M_W (32–400 × 10^3 g/mol). Lee & Mooney, (2012) and Gómez-Ordóñez et al. (2012b) identified alginates of 213–277 × 10^3 g/mol in *Saccharina latissima.* Thus, these peaks could be due to polyuronans with low-molecular weight described previously in brown

Table 2
M_W estimation of soluble polysaccharide fractions from the seaweeds *Himanthalia elongata* and *Gigartina pistillata* by HPSEC method.

Fraction	Yield (%)	Peak No.	Retention time (min)	Mw (x 10^3 g/mol)	Amount (mg/mL)
Himanthalia elongata					
F-H_2O	24.9	1	9.79 ± 0.03	926.44 ± 46.47^{d}	0.59 ± 0.05
F-HCl	2.1	1	10.22 ± 0.05	430.18 ± 41.16^{c}	0.13 ± 0.04
F-KOH	29.5	1	11.23 ± 0.03	67.85 ± 3.63^{b}	0.10 ± 0.01
		2	12.79 ± 0.02	3.85 ± 0.13^{a}	0.15 ± 0.01
Gigartina pistillata					
F-H_2O	53.0	1	9.05 ± 0.02	3617.25 ± 147.60^{e}	0.59 ± 0.03
F-HCl	10.6	1	9.43 ± 0.02	1813.43 ± 79.99^{d}	0.20 ± 0.09
		2	10.44 ± 0.06	286.91 ± 28.84^{c}	0.28 ± 0.06
F-KOH	8.4	1	12.05 ± 0.13	17.03 ± 5.44^{b}	0.16 ± 0.04
		2	13.05 ± 0.03	2.40 ± 0.13^{a}	0.40 ± 0.08

Yield (%) by gravimetry (g/100 g dry weight).
Different letters indicate significant ($p < 0.05$) differences between fractions for each seaweed.

Table 3
Monomeric composition (g/100 g dry weight) of polysaccharides fractions from *Gigartina pistillata*.

Fraction	Yield	Neutral sugars							UA	Total	Sulphate
		Rha	Fuc	Ara	Xyl	Man	Gal	Gluc			
F-H2O	55.2	–	–	–	–	–	65.5 ± 11.7^{d}	1.3 ± 0.9^{a}	1.3 ± 0.0^{c}	68.1	14.5 ± 1.3^{b}
F-HCl	9.5	–	–	–	–	–	47.2 ± 3.8^{c}	1.6 ± 0.3^{a}	0.9 ± 0.0b	49.7	16.3 ± 4.1^{b}
F-KOH	6.0	–	–	–	0.4 ± 0.1^{b}	0.4 ± 0.2^{a}	7.1 ± 0.9^{a}	8.2 ± 1.1^{b}	0.2 ± 0.0^{a}	16.3	2.7 ± 0.7^{a}
RES	3.7	–	–	–	0.2 ± 0.0^{a}	2.2 ± 0.3^{b}	16.6 ± 1.7^{b}	45.9 ± 3.5^{c}	1.0 ± 0.1^{b}	65.9	2.5 ± 0.2^{a}

Yield (%) by gravimetry (g/100 g dry weight). Total is the sum of neutral sugars and uronic acids.
Different letters indicate significant ($p < 0.05$) differences between fractions.

seaweeds (Gómez-Ordóñez et al., 2012b; Shevchenko et al., 2007). It is known that molecular weight of laminarans, fucoidans and alginates in brown algae also depend on the season of collection, the extraction method and/or the different species (Gómez-Ordóñez et al., 2012b; Rupérez et al., 2002).

3.2. Analysis of polysaccharide fractions from the seaweeds Gigartina pistillata

This red seaweed belongs to the Gigartinaceae family, which members are mainly carragenophytes. It was extracted in the same way than the brown seaweed *Himathalia elongata*. The extracted fractions and the residue (F-H_2O, F-HCl, F-KOH and RES) were analyzed through FTIR and the M_W and sugar composition were determined.

3.2.1. Determination of the monosaccharide composition

Table 3 show the sugar composition of the fractions obtained from this seaweed. Galactose is the most important sugar in all the fractions but RES. F-H_2O and F-HCl are mainly constituted of galactose and very little amounts of glucose and uronic acids. They are the most sulphated extracts. F-KOH presented similar contents of glucose and galactose, and RES is the richest fraction in glucose because of cellulose. All the fractions presented carrageenans, although those in F-H_2O and F-HCl are more sulphated than in the F-KOH and RES.

3.2.2. Structural analysis with fourier-transform infrared spectroscopy (FTIR)

The Fig. 2 shows the FTIR section between 2000 and 650 cm^{-1}. Fourteen bands are signed in the spectra. At 1210-1260 cm^{-1} appeared a broad band (Fig. 2, Band 4) in the standards and in *Gigartina,* more intense than in the soluble fractions from this seaweed analyzed. This band is ascribed to the S=O stretching vibration of the sulphated groups (Pereira Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009). The bands 5–7 at 1150-1010 cm^{-1} are related with C-O and C-C stretching vibrations of pyranose ring and they are signals very common to all the polysaccharides. The two more characteristics band in carrageenans are at 930 cm^{-1} (Fig. 2, Band 9) and 845 cm^{-1} (Fig. 2, Band 11) corresponding to 3,6-anhydrous-D-galactose and D-galactose-4-sulphate, respectively (Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009). Lambda-carrageenan presented a broader band (830-845 cm^{-1}) and a weaker band at 930 cm^{-1} than kappa-carrageenan as described Gómez-Ordóñez and Rupérez (2011). The band 8 at 960-970 cm-1 corresponds to galactose (Pereira et al., 2009), and showed high intensity in the standards. The band 9 appeared in the extracted fractions and the whole seaweed but it was very weak for F-HCl and F-KOH. The band 11 was not observed in F-KOH. The Gigartina seaweed provided consists on individuals from 3 different phases: female gametophytes, male gametophytes (non-fructified thalli) and tetrasporophytes. The gametophyte phases produce a hybrid kappa/iota carrageenan and the tetrasporophyte phase produces a csi (or xi)/lambda hybrid carrageenan (Pereira & Mesquita, 2003; Pereira, 2013). This would explain the presence of Lambda- and Kappa-carrageenans in the same seaweed.The band 10 at 900 cm^{-1} appeared just in lambda-carrageenan standard and is assigned to non-sulphated galactose (Pereira et al., 2009). The signal at 805 cm^{-1} (Fig. 2, Band 12) is related to the presence of sulphate in the 2-position of the anhydrous-D-galactose residues, a characteristic band of iota-carrageenan (Pereira et al., 2009). This band appeared more intense in lambda-carrageenan than in kappa-carrageenan standards but it was not observed in the fractions either in the whole seaweed. Therefore, the standards present a little content of iota-carrageenan but this polysaccharide is not present in *Gigartina*. The bands 13 and 14 at 720 and 700 cm^{-1}, respectively, are related to the skeleton bending of pyranose ring (Gómez-Ordóñez & Rupérez, 2011).

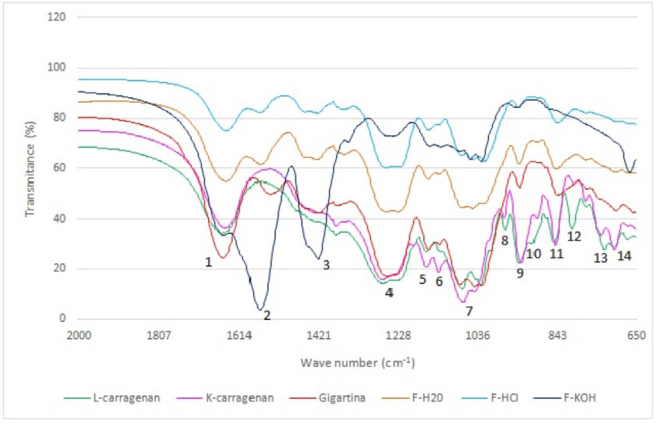

Fig. 2. Infrared spectra of polysaccharide standards: L-carragenan and K-carragenan and *Gigartina pistillata* fractions: whole *Gigartina pistillata*, water-extracted fraction (F-$H_2$0), hydrochloride-extracted fraction (F-HCl) and potassium hydroxide-extracted fraction (F-KOH) Band numbers (1–14) in FTIR spectra indicate most characteristic bands.

3.2.3. Molecular weight distribution

Retention time and molecular weight values estimated in *G. pistillata* soluble fractions are presented in Table 2. It is interesting to note that the molecular weight distribution of the soluble polysaccharides goes from the highest to the lowest values through the process of sequential extraction. Gómez-Ordóñez et al. (2012b) previously explained this behavior and it is because the polymerization degree of algal galactans varies significantly and is highly dependent on the extraction conditions. These authors also observed just one peak in each of their soluble fractions but in our study, we detected that F-H_2O exhibited a single peak, while F-HCl and F-KOH presented two peaks. Gómez-Ordóñez et al. (2012b) showed in *Mastocarpus stellatus* that the water-fraction peaks appeared at RT = 8.6 and 9.2, but we just observed the peak at this last RT, although with a greater M_W. The RT of F-HCl and F-KOH are lower than those from Gómez-Ordóñez et al. (2012b), and the M_W were also different. Commercial carrageenans have a molecular weight distribution ranging from 400 to 600 and 900 × 10^3 g/mol. The molecular weight distribution varies in the carrageenophytes seaweeds depending on age of the harvested seaweed, season of harvesting, way

Table 4
pH, gas and Short Chain Fatty Acids (mmol/g) production during the *in vitro* fermentation of the polysaccharides fractions from *Himanthalia elongata* and *Gigartina pistillata*.

	pH	Gas (psi)	Short Chain Fatty Acids (SCFA)						
			Acetic	Propionic	Butyric	Isobutyric	Isovaleric	Valeric	Total SCFA
Himanthalia elongata									
F-H_2O	7.1 ± 0.0	2.0 ± 0.0	5.1 ± 0.8^{b*}	1.2 ± 0.5^{a*}	1.3 ± 0.2*	1.7 ± 0.5^{b*}	1.1 ± 0.2^{b*}	1.3 ± 0.2^{b*}	11.8 ± 2.4^{b*}
F-HCl	7.0 ± 0.1	–	3.6 ± 0.5^{a}	1.6 ± 0.1^{a*}	1.6 ± 0.3*	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.6 ± 0.1^{a}	8.2 ± 0.9^{a}
F-KOH	7.1 ± 0.1	–	3.0 ± 0.9^{a}	1.3 ± 0.3^{a}	1.5 ± 0.4*	1.9 ± 0.6^{b*}	1.4 ± 0.5^{b*}	1.3 ± 0.4^{b*}	10.4 ± 1.8^{ab}
RES	6.9 ± 0.1	4.0 ± 1.0	5.7 ± 0.5^{b*}	2.3 ± 0.2^{b*}	1.4 ± 0.1	0.4 ± 0.1^{a}	0.4 ± 0.0^{a}	0.6 ± 0.0^{a}	11.6 ± 2.1^{b*}
Gigartina pistillata									
F-H_2O	7.1 ± 0.1	–	2.5 ± 0.3^{a}	0.7 ± 0.2^{a}	0.7 ± 0.3^{a}	0.8 ± 0.1^{b}	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}	5.3 ± 0.6^{a}
F-HCl	7.1 ± 0.0	–	3.4 ± 0.2^{b}	1.2 ± 0.1^{b}	0.9 ± 0.1^{ab}	0.5 ± 0.1^{a}	0.6 ± 0.1^{b}	0.7 ± 0.1^{b}	8.3 ± 1.7^{b}
F-KOH	7.1 ± 0.0	–	3.4 ± 0.2^{b}	1.4 ± 0.1^{bc}	1.1 ± 0.1^{b}	0.7 ± 0.1^{b}	0.8 ± 0.1^{c}	0.7 ± 0.1^{b}	7.9 ± 0.7^{b}
RES	7.0 ± 0.0	–	3.9 ± 0.1^{b}	1.6 ± 0.1^{c}	1.1 ± 0.1^{b}	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.5 ± 0.0^{a}	7.9 ± 0.1^{b}

Different letters indicate significant ($p < 0.05$) differences between fractions for each seaweed.
* indicates significant ($p < 0.05$) difference for the same fraction between both seaweeds.

of extracting and length of heat treatment (Tuvikene et al., 2010).

3.3. Evaluation of the in vitro fermentation of the seaweeds polysaccharides fractions

The colonic microbial activity is usually evaluated by measuring metabolic end products such as SCFA and gases. Table 4 shows pH, gas production and the concentrations of SCFA after 24 h of *in vitro* fermentation. There were not changes in pH measurement. The gas production was only detected in lactulose and in F-H_2O and RES from *Himathalia elongata*. Determination of SCFA production provided an estimation of the degree of substrate fermentation by the caecal microbiota. Acetic acid was the main SCFA produced and it was larger ($p < 0.05$) from the fermentation of F-H_2O and RES from *Himathalia elongata*. Propionic acid increased ($p < 0.05$) in RES from this seaweed. Butyric acid was in similar amounts for all fractions from *Himanthalia*. A part of the produced SCFA were branched SCFA (BSCFA); the sum of isobutyric, isovaleric and valeric acids represented 35%, 17%, 44% and 12% for F-H_2O, F-HCl, F-KOH and RES from *Himanthalia*, respectively, and in the case of *Gigartina*: 32%, 22%, 28% and 18% for F-H_2O, F-HCl, F-KOH and RES, respectively. The production of BSCFA could be related with the catabolism of resistant protein (McFarlane, Gibson, Beatty, & Cummings, 2006), indeed a remarkable content of nitrogen, quantified by Kjedahl method, was found in the fractions and the whole seaweed *Himanthalia* (F-H_2O: 3.3%; F-HCl: 5%; F-KOH: 6%; RES: 5.2%) and *Gigartina* (F-H_2O: 1.7%; F-HCl: 5.6%; F-KOH: 7.5%; RES: 5%). In general terms, the obtained polysaccharides fractions produced more BSCFA than lactulose, especially F-H_2O and F-KOH fractions from *Himanthalia* and *Gigartina*.

The fermentability of the fractions from *Himanthalia*, calculated from the total production of SCFA (referred to lactulose as 100%), was low and only F-H_2O, F-KOH and RES exceeded 30% fermentability respect to lactulose. An increase ($p < 0.05$) of total SCFA was found in the water and hydrochloride fractions that contains laminarans, that are considered as fermentable substrates (Kuda, Yano, Matsuda, & Nishizawa, 2005; O'Sullivan et al., 2010). Presumably, the amount of laminarans is higher in F-HCl than in F-H_2O. However a significant major ($p < 0.05$) concentration of SCFA was found in F-H_2O.This fact could be due to the high BSCFA production found in this fraction, and also because of the potential fermentability of the extracted fucans (different to fucoidans), since they are not described as prebiotic compounds in the literature (O'Sullivan et al., 2010). Therefore, the fucans xylofucoglycuronans or glycuronogalactofucans, and xylomannans, possibly presented in this fraction, could be SCFA producing substrates. The alginates and/or polyuronans described in F-KOH with M_W about 68 and 4×10^3 g/mol, seem to be less suitable for fermentation than heavier ones, indicating that molecular weight seems not to have a great impact in the prebiotic effect as Ramnani et al. (2012) highlighted, and maybe the chemical structure and the sugar composition seem to be more determinant. This is remarkable because different studies on plant polysaccharides have indicated that low molecular weight oligosaccharides increase fermentability by gut microbial communities (Ho, Kosik, Lovegrove, Charalampopoulos, & Rastall, 2018; Hughes, Shewry, Gibson, McCleary, & Rastall, 2008; Yang et al., 2017), and also it has been observed with oligosaccharides derived from chitosan (Mateos-Aparicio, Mengíbar, & Heras, 2016). All SCFA and gas production were found increased ($p < 0.05$) in RES from *Himanthalia,* highlighting the fermentation of this cellulose-rich fraction, probably because of the presence of non-extractable glycuronogalactofucans and xylomannans (Table 1), appearing in strong association with cellulose (Mateos-Aparicio et al., 2010).

The fermentability of the polysaccharides from *Gigartina* was 13–16% respect to lactulose and a notable part of the SCFA produced were branched from the fermentation of nitrogenous material. Previous studies with Wistar rats showed no clear or non-effect over the red seaweeds *Mastocarpus stellatus* and *Gigartina pistillata* (Gómez-Ordoñez et al., 2012a; Villanueva et al., 2014). Indeed, the red seaweed Nori was fermented by *Bifidobacterium* strains just in presence of high protein content (Muraoka et al., 2008; O'Sullivan et al., 2010). Ramnani et al. (2012) found a derived agar from the red seaweed *Gelidium sesquipidale* with Mw of 64×10^3 g/mol able to ferment *in vitro* showing a significant increase in bifidobacterial populations. Therefore, the polysaccharides from agarphytes red seaweeds appear as potential fermentable by intestinal microbiota and the carrageen from the carragenophytes red ones do not seem to present this effect.

Considering both seaweeds rich in DF and soluble polysaccharides, a higher SCFA production could have been expected, at least for the polysaccharides from *Himanthalia*, because its intake may increase SCFA production in Wistar rats (Villanueva et al., 2014). However, the chemical structure, composition and molecular weight of algal polysaccharides affect their fermentative behaviour, and the entire matrix could be more fermentable than their polysaccharides fractions. Similarly, the development of antioxidant activity of oligosaccharides from *Himathalia elongata* depend on oligosaccharide type, structure and/or spatial pattern of sulphate groups (Mateos-Aparicio, Molina, & Redondo-Cuenca, 2015).

4. Conclusions

Fucoidans and, probably, water-soluble alginates and xylofucoglycuronans with high estimated molecular weights ($930–430 \times 10^3$ g/mol) were better extracted with hot water than with mild acid conditions. In contrast, laminarans were found in major amount in the hydrochloride fraction than in the aqueous one from *Himanthalia elongata*.

Low molecular weight polyuronans (4–68 × 10^3 g/mol) were extracted mainly with KOH. The polymerization degree of carrageenans from *Gigartina pistillata* was lowered with the sequential extraction. 24 h may not has been enough to allow further breakdown of the complex polysaccharides, very different to those of the plant polysaccharides. Their structures, sugar composition, molecular weight and other fermentable substrates, such as resistant protein, can induce different fermentative patterns. In fact, an increase of BCFA was observed in every fraction due to the presence of some peptides or proteins and low production of acetic, propionic and butyric acids, indicating the poor fermentability of the polysaccharides carrageenans from *Gigartina pistillata,* polyuronans and fractions rich in laminarans and fucoidans from *Himanthalia elongata*. However, the xylofucoglycuronan and xylomannans extracted with water or remaining in the last residue from *Himanthalia* seems to be more fermentable. These findings are supported by previous studies that pointed towards the carragenophytes red seaweeds not being fermentable whereas brown algae present a higher fermentability, probably due to the presence of laminarans, xylofucoglycuronans and/or xylomannans. The low fermentability found in the *in vitro* analysis compared with *in vivo* analysis previously carried out may be due to the fact of the entire matrix being more fermentable than their extracted polysaccharides.

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