



## Characterization and gelling properties of a bioactive extract from *Ascophyllum nodosum* obtained using a chemical-free approach



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### ABSTRACT

The bioactivity and gelling properties of a carbohydrate-rich algal extract obtained from locally harvested *Ascophyllum nodosum* seaweed using a chemical-free approach were investigated for its potential interest in food applications. Physicochemical characterisation and compositional analysis of the extract, using FTIR, biochemical methods and monosaccharide analysis, confirmed the presence of alginates and fucoidans, although the main polysaccharide present in it was laminarin. Significant amounts of phenolic compounds (~9 mg phloroglucinol/100 mg sample) were also detected. As a result, the extract exhibited good antioxidant activity. It also showed promising prebiotic potential, promoting the growth of beneficial *Lactobacillus* sp. and *Bifidobacteria* sp. when compared with commercial prebiotics, but not that of pathogenic bacteria such as *E. coli* or *P. aeruginosa*. The gelling properties of the raw extract were explored to optimize hydrogel bead formation by external gelation in CaCl<sub>2</sub> solutions. This was enhanced at neutral to alkaline pHs and high extract and CaCl<sub>2</sub> concentrations. The mechanical strength, nano- and microstructure of the hydrogel beads prepared under optimised conditions were determined using compression tests, synchrotron small- and wide-angle X-ray scattering (SAXS/WAXS) and scanning electron microscopy (SEM). It was concluded that the raw algal extract at neutral pH had potential for use as a gelling agent, although further enrichment with alginate improved the mechanical properties of the obtained gels. The advantages and disadvantages of applying the non-purified algal extract in comparison with purified carbohydrates are discussed.

### 1. Introduction

Seaweeds are an abundant, natural and renewable source of polysaccharides for use in the food, feed, pharmaceutical and cosmetics industries. Algae-derived polysaccharides have unique structural and functional properties (Foley et al., 2011). Among them, alginates are the group of algal polysaccharides most used in industry (Roohinejad et al., 2017), and have been extensively used as food-grade and biocompatible

encapsulation matrices (Philippe E Ramos et al., 2018) due to their gelling properties and pH sensitivity, which can be exploited for targeted release applications (Agüero et al., 2017). Alginates are extracted from the cell walls of brown algae (L.-E. Rioux and Turgeon, 2015) and their structure consists of (1 → 4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues randomly distributed along the carbohydrate chains (de Celis Alonso et al., 2010), forming M-blocks (consecutive M residues), G-blocks (consecutive G residues) and

**Abbreviations:** AAE, ascorbic acid equivalents; ATR, attenuated total reflectance; BSA, bovine serum albumin; FOS, fructooligosaccharides; FTIR, Fourier transform infrared spectroscopy; G, α-L-guluronic acid; GOS, galactooligosaccharides; M, β-D-mannuronic acid; NCF, protein conversion factor; OD, optical density; PGE, phloroglucinol equivalents; SAXS, small-angle X-ray scattering; SEM, scanning electron microscopy; TE, Trolox equivalents; WAXS, wide-angle X-ray scattering.

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MG-blocks (alternating M and G residues) (García-Vaquero, Rajauria, O'Doherty and Sweeney, 2017). The G residues of alginates can bind multivalent cations, offering the possibility of producing alginate hydrogel beads at room temperature by ionic crosslinking (Liu et al., 2016; Strobel et al., 2016). Due to its biocompatibility and non-toxicity,  $\text{Ca}^{2+}$  is most often selected as a gelling cation for alginates (Agüero et al., 2017; Sun and Tan, 2013).

To date, most applications that exploit alginate as a gelling agent make use of purified alginate grades (Bannikova et al., 2018; Li et al., 2016; Martins et al., 2017; Nikoo et al., 2018; Santa-Maria et al., 2012; Shaharuddin and Muhamad, 2015; Wikström et al., 2008). However, the purification of seaweed polysaccharides from raw algal extracts usually involves multiple energy- and time-consuming steps, which also require the use of large volumes of water (Bixler and Porse, 2011; McHugh, 2003). Moreover, unpurified seaweed extracts usually contain functional compounds such as polyphenols (Alehosseini et al., 2018; Cardozo et al., 2007) that are frequently lost during purification processes (Martínez-Sanz et al., 2019). In this context, using raw seaweed extracts rather than purified polysaccharides could be an advantageous alternative, since apart from the economic savings and environmental benefits, the additional natural and potentially bioactive compounds found in the raw extracts may add extra value to the final products. In support of this concept, some unpurified extracts from seaweeds and other sources of aquatic biomass, obtained using simplified extraction protocols, have been found to exhibit enhanced performance, including improved mechanical properties (Martínez-Sanz et al., 2018), antioxidant capacities (Blanco-Pascual et al., 2014) and a superior protective effect on sensitive probiotic bacteria (Alehosseini et al., 2018).

The industrial process for the extraction of alginates generally involves an initial acid pre-treatment to transform the alginate salts present in the algal cell walls into alginic acid, followed by an alkali extraction step in which alginic acid is transformed into water-soluble sodium alginate, which is then separated from the solid residues by flotation/flocculation and filtration. In order to purify the extracted alginate, it is then precipitated and separated from the liquid fraction before drying (Blanco-Pascual et al., 2014; Vauchel et al., 2009). Foley et al. (2011) proposed an alternative low-chemical approach for the simultaneous extraction of alginate and fucoidan fractions from *Ascophyllum nodosum*. This approach included a pre-treatment with ethanol 80% to eliminate mannitol and some of the salts, followed by an extraction in water that avoided the use of acid and alkali treatments, which are known to result in the depolymerisation of alginates (Peggy et al., 2008). Using water as the sole and only solvent for extraction of polysaccharides has been explored previously to obtain less purified agar extracts from red seaweed (Martínez-Sanz et al., 2019), and is proposed in this work as an alternative approach for the extraction of carbohydrates from the brown algae *A. nodosum*. It is worth mentioning that, although extraction in hot water still involves the input of water and energy to the system, the conventional industrial processes also require these inputs, plus the use of harsh chemicals with their subsequent safety and environmental concerns.

The fucoidans and alginate-rich fractions produced by Foley et al. (2011) were subsequently separated by precipitation of the alginates with calcium chloride (Foley et al., 2011). However, the valorisation of raw, unpurified extracts is also considered of practical interest, as commented on above. The presence of fucoidans in a raw extract may add value, since this group of polysaccharides rich in fucose has been reported to offer several health benefits, including anti-coagulant, anti-proliferative and anti-inflammatory activities (Senthilkumar et al., 2013; Vo and Kim, 2013; Wijesinghe and Jeon, 2012). The structure of fucoidans is very heterogeneous and depends strongly on the species and extraction method, but it is generally accepted that they mainly consist of a linear or branched backbone of  $\alpha$ -(1–3) or alternating  $\alpha$ -(1–3) with  $\alpha$ -(1–4)-linked L-fucopyranoside, whose subunits can be mono- or di-sulphated or acetylated, and can also contain mannose, xylose, galactose and/or glucose, and uronic acids (Zayed and Ulber, 2019).

Since alginates are one of the most abundant components of *A. nodosum* (Rioux et al., 2007; Yuan and Macquarrie, 2015), algal extracts obtained from this edible seaweed might be expected to exhibit good gelling properties. Therefore, their exploitation as hydrogel-forming matrices for food applications could be a plausible option for their valorisation, as already proposed for other non-purified algal extracts such as agar-based fractions obtained from *Gelidium sesquipedale* (Alehosseini et al., 2018). On the other hand, the successful production of composite alginate/fucoidans hydrogel beads by external gelation methods has been previously reported using a mixture of purified alginate and fucoidans (Karunanithi et al., 2016). However, from a practical point of view, using a raw algal extract containing both alginates and fucoidans would be a more attractive alternative than blending previously purified polysaccharides.

In this work, a carbohydrate-rich extract was obtained from *A. nodosum*, using water as the only solvent for extraction. Its prebiotic and antioxidant activities were explored, as well as its gelling properties under different conditions. The impact of extract composition and structure on the physicochemical, rheological and mechanical properties of hydrogel beads was studied, in order to determine the best conditions for their exploitation. For this purpose, a common extrusion-external gelation technique, which consists of extruding the matrix solution through an orifice into a bath containing a gelling agent (Whelehan and Marison, 2011), was used. A commercial alginate sample was also evaluated for comparative purposes. The relevance of this work relies on the use of a very simple, mild extraction procedure to produce a complex algal extract containing not only alginate but also other bioactive compounds, and on the in-depth study of how the presence of those additional components affected functional properties of alginate such as its gelling properties.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Algal material

*A. nodosum* was harvested from Dungloe Bay, Co. Donegal, Ireland, in September 2012. The seaweed was washed with fresh water, cut into pieces (ca. 1 mm<sup>2</sup>) and stored at –20 °C until further use.

#### 2.1.2. Chemicals

Sodium alginate from *Laminaria hyperborea* and potassium ferricyanide were obtained from BDH Laboratory Supplies (England). Ammonium molybdate, ethanol, ferric chloride, phenol, sodium chloride, sodium sulphate anhydrous, sulfamic acid, trichloroacetic acid and Trolox were purchased from Fisher Scientific (Ireland). D-mannuronic acid and L-guluronic acid were purchased from Carbosynth (UK). Vivinal GOS was a gift from Dr. Shane O'Connell (Institute of Technology Tralee, Ireland). All other reagents were obtained from Sigma-Aldrich (Ireland). Bacterial cultures were purchased from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany) and from the NCIMB (National Collection of Industrial, Food and Marine Bacteria, UK).

### 2.2. Production of the algal extract

The algal extract was prepared by extraction in hot water. Finely cut *A. nodosum* pieces were extracted at a ratio of 33% wet weight/volume with warm water (70 °C–100 °C) for 6 h. The liquid (aqueous) fraction was then processed by ultrafiltration through three different molecular size cut-off membranes (from 1 kDa to 10 kDa), collecting the retentate after each step, to reduce the water content. The last retentate was then spray-dried to obtain a uniform powder. The yield of extraction was 17.3% (grams of total solids obtained per 100 g of dry seaweed extracted).

### 2.3. Chemical composition of the algal extract

#### 2.3.1. Carbohydrate composition

Total carbohydrate content was determined using a microassay version of the Dubois assay (DuBois et al., 1956). Quantification was achieved using glucuronic acid as standard. The uronic acid content was quantified based on the method of Filisetti-Cozzi and Carpita (1991), using uronic acid (glucuronic acid or mannuronic acid) as standards. The fucoidan content was estimated following the method used by Saboural et al. (2014). A commercial fucoidan from *F. vesiculosus* was used as standard. The sulphate content was evaluated using the BaCl<sub>2</sub> turbidimetric method described by Rossum and Villarruz (1961) and sodium sulphate anhydrous as standard. Free D-mannitol was determined enzymatically using the K-MANOL kit (Megazyme, Bray Co. Wicklow, Ireland) according to the manufacturer's instructions. Each sample was analysed in triplicate.

The monosaccharide composition of the extract was further determined in duplicate after acid methanolysis. Samples (1 mg) were incubated with 1 mL of 2 M HCl in dry methanol for 5 h at 100 °C, then neutralized with pyridine, dried under a stream of air, and further hydrolysed with 2 M TFA at 100 °C for 1 h. The samples were dried again under a stream of air and dissolved in water. Monosaccharides were analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex).

#### 2.3.2. Protein content

Total soluble protein was quantified (in triplicate) according to a modification of the method of Lowry (Bensadoun and Weinstein, 1976), using BSA as standard. The total protein content was determined using a Leco FP-528 N Analyzer (Leco Instruments UK Ltd., Cheshire, UK). While there are different reports on the nitrogen to protein conversion factors (NCF) of algae (Biancarosa et al., 2017), a universal NCF of 6.25 was used similar to other studies (Martínez-Sanz et al., 2018; Yuan Yuan and Macquarrie, 2015).

#### 2.3.3. Total phenolics

The content of phenolic compounds was estimated according to the method described by Zhang and co-workers (Zhang et al., 2006), using phloroglucinol as a reference. This was assessed on 1% w/v dispersions of the extract. To account for the interferences of reducing compounds in this method, the dispersion was also analysed after peroxidation with 1.5 M H<sub>2</sub>O<sub>2</sub> at a volumetric ratio of 1:1 (Sánchez-Rangel et al., 2013). Additionally, an alternative method reported by Medina (2011) was used to quantify the content of total phenolics. All the standards and samples were analysed in triplicate.

#### 2.3.4. Reducing sugars

The content of reducing sugars was analysed in triplicate following the method described by Miller (1959). Quantification was done using glucose as standard.

#### 2.3.5. Total lipids

The total lipid content of the extract was determined in triplicate using the gravimetric method described by Bligh and Dyer (1959).

#### 2.3.6. Moisture and ash content

The moisture and ash content of the samples were analysed in duplicate using a Leco TGA701 gravimetric oven (Leco Instruments UK Ltd., Cheshire, UK). The samples (ca. 1 g) were first heated from 25 °C to 102 °C at a heating rate of 1 °C/min and maintained at 102 °C for 4 h to determine the moisture content. The dry samples were then subjected to a second heating ramp from 102 °C to 550 °C at a heating rate of 15 °C/min, and maintained at 550 °C for 4 h, to determine the ash content.

#### 2.3.7. Mineral analysis

The mineral profile of each sample was determined in duplicate after ashing, as described above. The ashed samples were first dissolved in 1 mL of 1 M nitric acid and subsequently diluted 10<sup>4</sup>-fold with 2 mM nitric acid. The resulting solutions were filtered using 0.2 µm polysulfone syringe filters before analysis, and their mineral content was determined as described previously (Murphy et al., 2018), using a Dionex ICS-5000+ Ion Chromatography system (Thermo Fischer Scientific, Dublin, Ireland). Cations were separated using a CS16-4 mm column under isocratic conditions with methanesulfonic acid used for eluent generation, and anions were separated using an AS11-HC column under gradient conditions, with KOH used for eluent generation.

Additional physico-chemical analyses performed on the extract can be found in Supplementary Material.

### 2.4. Antioxidant activity

The total antioxidant activity of the extract was evaluated in triplicate using the method from Prieto et al. (1999). Results were expressed as milligram of Trolox equivalents (TE) and ascorbic acid equivalents (AAE) per gram of dry sample.

The DPPH radical-scavenging activity of the extract was evaluated using the method described by Guerra Dore et al. (2013). Results were expressed as the half maximal (EC<sub>max</sub>) effective concentration (EC<sub>50</sub>), i.e. the amount of sample necessary to decrease the absorbance of DPPH by 50%. Trolox was used as reference and all the standard and samples were analysed in triplicate.

The reducing power of the extract was evaluated in triplicate by the method reported by Kuda et al. (2005). Results were expressed as mg of Trolox equivalents (TE), ascorbic acid equivalents (AAE) and phloroglucinol equivalents (PGE) per g of sample.

### 2.5. Antimicrobial and prebiotic activities

The antimicrobial and prebiotic activities of the extract were tested by evaluating the growth response of different bacterial strains (*B. angulatum*, ATCC 27670; *E. coli*, ATCC 25922; *L. acidophilus* ATCC 4356; *L. casei*, ATCC 393; *L. fermentum* ATCC 14931; *L. gallinarum*, ATCC 33199; *L. plantarum*, ATCC 14917; *L. reuteri*, ATCC 3272 and *P. aeruginosa*, ATCC 10145) to the algal extract which was supplied as only carbon source in comparison to commercial carbon sources. A single colony was transferred to 25 mL of the appropriate sterilised broth medium (M9 for *E. coli* and *P. aeruginosa*, and MRS for the probiotic strains), and incubated at 37 °C overnight, with shaking at 140 rpm, under aerobic conditions, to obtain seed cultures.

For each test, 20 µL of seed bacterial culture were used to inoculate 180 µL of M9 or MRS medium containing 0.1% or 0.3% w/v of the extract as the only carbon source, in four replicate wells in sterile 96-well microplates. Glucose and two commercial prebiotics (fructooligosaccharides, FOS, and galactooligosaccharides, GOS) were used as positive controls. The media with no added carbon source, plus the media without bacteria, were used as negative controls. Microplates were incubated at 37 °C for 24 h and the optical density (OD) of the cultures was evaluated every 30 min at 595 nm using the Gen5 software in kinetic mode (BioTek Powerwave XS2).

The growth rate (Eq. (1)) was defined as the variation in population density (triplicate cultures) occurring in the conditions tested for the extract relative to the commercial prebiotics. Positive values indicate a promotion of bacterial growth when supplied with the algal extract in comparison to the controls FOS and GOS. Negative values indicate that the algal extract inhibited the bacterial growth when compared with these controls.

$$\text{Growth rate (\%)} = 100 \left( \left( \frac{OD_S - OD_C}{OD_C} \right) \right) \quad \text{Eq. (1)}$$

**Table 1**Estimated proximal composition of the algal extract obtained from *Ascophyllum nodosum*.

Dry solids (g/kg)	962 ± 3
Carbohydrates (g/kg)	398 ± 10 <sup>a</sup> /393 ± 47 <sup>b</sup> 0.2
Proteins (g/kg)	22 ± 2 <sup>c</sup> /59 ± 1 <sup>d</sup>
Lipids (g/kg)	1.97 ± 0.01
Phenolics (g phloroglucinol/kg)	102.1 ± 0.1 <sup>e</sup> /88.6 ± 0.3 <sup>f</sup> /98.1 ± 0.1 <sup>g</sup>
Reducing sugars (g glucose/kg)	59.2 ± 0.4
Sulphates (g sulphate/kg)	23 ± 1
Fucoidan (g fucoidan/kg)	72 ± 0 <sup>h</sup> /43 ± 2 <sup>b</sup>
Alginate (g uronic acids/kg)	86 ± 0 <sup>h</sup> /17 ± 0 <sup>b</sup>
Mannitol (g D-mannitol/kg)	108 ± 1 <sup>a</sup> /98 ± 15 <sup>b</sup>
Moisture (g/kg)	38 ± 3
Ash (g/kg)	298 ± 1
Cations (g/kg)	
Lithium	-
Sodium	51.3 ± 1.0
Ammonium	-
Potassium	41.2 ± 2.1
Magnesium	1.9 ± 0.2
Calcium	2.2 ± 0.3
Anions (g/kg)	
Chloride	2.44 ± 0.04
Sulphate	1.42 ± 0.02
Phosphate	0.064 ± 0.004

<sup>a</sup> According to the specific colorimetric method.<sup>b</sup> according to HPAEC-PAD analysis (fucoidan is calculated as the sum of Fuc, Xyl and GlcA units, alginate as the sum of ManA and GulA units, and the total carbohydrate content as the sum of all monosaccharide units).<sup>c</sup> according to the Lowry method.<sup>d</sup> calculated from the total nitrogen content using a NCF of 6.25.<sup>e</sup> according to the method by Zhang et al. (2006) without sample peroxidation.<sup>f</sup> according to the method by Zhang et al. (2006) after sample peroxidation.<sup>g</sup> according to the method by Medina (2011).

where OD<sub>C</sub> is the mean optical density of the controls; OD<sub>S</sub> is the mean optical density of the tested extract sample.

## 2.6. Rheological properties

The viscosity of the extract suspensions was studied using a rheometer model AR-2000 (TA Instruments, USA) with a parallel plate geometry (60 mm diameter and 500 μm gap) following a procedure adapted from (Gómez-Mascaraque et al., 2015). Continuous shear rate ramps were performed from 0.1 to 500 s<sup>-1</sup> over 10 min, at 25 °C, following pre-shear treatment at 1s<sup>-1</sup> for 10 s and equilibration for 1 min. All measurements were made at least in triplicate.

## 2.7. Gelling capacity of the extract

Dispersions of the algal extract (20% and 50% w/v) and calcium chloride solutions (0.2–1 M), both in distilled water, were prepared at 40 °C and room temperature, respectively, under magnetic agitation. The pH of the extract dispersions was adjusted from pH 4.5 to 13.0 with 2 M NaOH. The dispersions (200 μL aliquots) were then added drop-wise to glass beakers containing 2.5 mL of the CaCl<sub>2</sub> solutions using transfer pipettes and the formation of extract hydrogel beads was evaluated.

## 2.8. Mechanical properties of the hydrogel beads

Uniaxial compression tests were performed at room temperature using a texture analyzer (Stable Micro Systems model TA-XTplus, Surrey, UK). The hydrogel beads were carefully placed forming a tightly packed monolayer on a piece of tape to avoid slippage. A cylindrical aluminium probe with a diameter of 25 mm was then used to compress the beads at a compression rate of 0.1 mm/s to a maximum strain of 70%, using a 5 kg load. All measurements were performed in triplicate.

## 2.9. Scanning electron microscopy (SEM)

SEM was conducted on a Gemini field emission scanning electron microscope (ZEISS, Germany) at an accelerating voltage of 2–7 kV and a working distance of 9–25 mm after sputter-coating the samples with chromium using an Emitech K575X sputter coater (Quorum Technologies, UK).

## 3. Results and discussion

A pink/brown algal extract in the form of a fine powder was obtained using a simple hot water extraction method followed by spray-drying. Figs. S1A and B of the Supplementary Material show SEM micrographs of the spray-dried algal extract.

### 3.1. Composition of the algal extract

Table 1 summarises the main components in the algal extract, while the complete carbohydrate composition is shown in Table S1 of the Supplementary Material. A complete FT-IR analysis can also be found in the Supplementary Material (Fig. S2). It is worth mentioning that, due to complexity of unpurified algal extracts, most of the methods used to characterise their proximal composition provide only estimated results. This is especially the case of the colorimetric methods, in which the presence of other compounds may interfere in the quantification of the compounds of interest. For this reason, two different methods have been used for the estimation of each type of components where possible.

Carbohydrates are the most abundant components of *A. nodosum* species, accounting for about 45% of their mass (Yuan and Macquarrie, 2015). The main carbohydrate components in the extract were found to be laminarin and mannitol, accounting for around 65% and 27% of all carbohydrates, respectively. The absence of purification steps to remove mannitol or purify the fucoidans or alginates, explains these relatively high quantities, as opposed to previously reported methods (Foley et al., 2011; L. E. Rioux et al., 2007). *A. nodosum* is known to accumulate laminarin and mannitol in the early autumn as an energy reserve through the winter (Manns et al., 2014). The harvesting period of the algae (September) further explains these relatively high quantities. Alginate and fucoidan accounted for about 5–15% and 10–20%, respectively, of the total carbohydrate content, depending on the quantification method (Table 1).

The main minerals present in *A. nodosum* are reported to be sodium, sulphur, magnesium, calcium, potassium and phosphorus (Yuan and Macquarrie, 2015). All of these mineral elements (plus chloride) were detected in significant amounts in the algal extract. The total phenolic content of the extract was much higher than that reported for short (30 min) extractions of *A. nodosum* in hot (80 °C) water performed over a full year, with values ranging from 6.8 (mg gallic acid equivalents/g) (June) to 2.6 (mg gallic acid equivalents/g) (April) (Apostolidis and Lee, 2010; Apostolidis et al., 2011). It was also higher than those reported for extracts from other brown seaweed such as *Sargassum marginatum* (24.61 gallic acid equivalents/1000 mg extract) and *Padina tetrastomatica* (20.04 gallic acid equivalents/1000 mg extract) (Chandini et al., 2008). Additionally, the extract contained around 2–5% protein. All these impurities could be expected for an unpurified seaweed fraction extracted in water (Martínez-Sanz et al., 2019). The aqueous-based extraction also explains its low lipid content.

### 3.2. Antioxidant activity of the algal extract

Brown algae exhibit a high content of phlorotannins, which are regarded as the main components responsible for the antioxidant properties of extracts from *A. nodosum* (Audibert et al., 2010; Connan et al., 2004) and show higher radical potential scavenging than phenolics derived from terrestrial plants (Ahn et al., 2007). Fucoidans have also been reported to have antioxidant activities that vary depending on their

**Table 2**Antioxidant activity of the polysaccharide rich algal extracted from *Ascophyllum nodosum*, determined by DPPH, reducing power and total antioxidant assays.

Sample	Total antioxidant activity		Reducing Power			DPPH Assay	
	TEAC (mg TE/g sample)	AAEC (mg AAE/g sample)	TEAC (mg TE/g sample)	AAEC (mg AAE/g sample)	PGE (mg PGE/g sample)	EC <sub>50</sub> (mg/ml)	EC <sub>max</sub> (mg/ml)
Sample	1760 ± 77	436 ± 28	4400 ± 260	1557 ± 100	1117 ± 69	0.095 ± 0.002	0.501 ± 0.004
Trolox					-	0.076 ± 0.001	0.175 ± 0.003

Abbreviations: TE, Trolox equivalents; AAE, ascorbic acid equivalents; EC<sub>50</sub>, half effective concentration; EC<sub>max</sub>, maximal effective concentration, PGE, phloroglucinol equivalents.

size and structure (J. Wang, Zhang, Zhang, Song and Li, 2010; Xue et al., 1998).

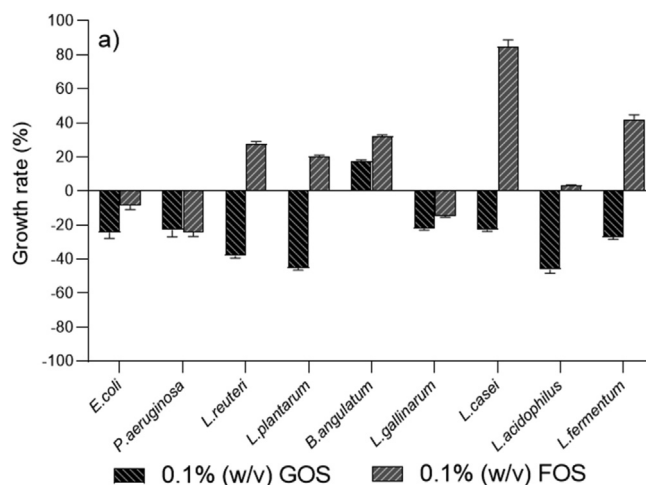
The total antioxidant activity for the *A. nodosum* extract produced in this work (Table 2) was higher than that reported for *T. conoides* (9.65 mg AAE/g extract), and its DPPH radical scavenging activity was almost five times higher than those reported for *T. conoides* (12.69 ± 0.74%), *Sargassum marginatum* (2.97 ± 0.74%), and *Padina tetrastomatica* (2.92 ± 0.47%) (Chandini et al., 2008). Based on these results, this algal extract could potentially be used as source of natural antioxidants.

### 3.3. Prebiotic and antimicrobial potential of the algal extract

The prebiotic properties of certain polysaccharides present in brown seaweed have been reported in recent years. For instance, fucoidan can stimulate growth of *Bifidobacteria* sp. (Zaporozhets et al., 2014), *Lactobacillus* sp. and *Ruminococcaceae* sp. (Shang et al., 2016), both *in-vivo* and *in-vitro*. To assess the prebiotic potential of the algal extract from obtained in this work, the growth of different probiotic bacteria in the presence of the extract was assessed in comparison with two commercial prebiotic preparations, i.e. FOS and GOS. The results for two different concentrations of the extract, i.e. 0.1% and 0.3% (w/v), are presented in Figs. 1 and S9, respectively.

The extract from *A. nodosum*, was not as effective as GOS in promoting growth of most of the strains of probiotic bacteria assessed. Supplementation with the extract at 0.1% only increased the growth rate of *B. angulatum* when compared to GOS. However, the growth of most of the probiotic strains was promoted when compared to FOS: *L. acidophilus*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri* and *B. angulatum*. The behaviour was very similar when the algal extract was applied at 0.3% (Fig. S9), which confirmed its prebiotic properties. Although at this concentration only the growth rate of *L. gallinarum* increased using the algal extract in comparison with GOS, the growth of most of the probiotic microorganisms was better promoted by the algal extract than by FOS, except that of *B. angulatum*.

The activity of the algal extract was also tested against *E. coli* and *P. aeruginosa*, to confirm that it did not promote the growth of these pathogenic bacteria. The supplementation with the algal extract at the two concentrations tested resulted in a slower growth rate of both *E. coli* and of *P. aeruginosa* when compared to the commercial prebiotics FOS and GOS. This is consistent with previous works that reported antimicrobial activities for some algal components such as fucoidans or polyphenols. A number of phlorotannins isolated from brown algae have shown antimicrobial effects against food-borne pathogens (Ahn et al., 2004; Eom et al., 2012). In particular, phlorotannin extracts from *A. nodosum* were reported to have bactericidal effects against *E. coli* (Wang et al., 2009). The activity of fucoidans against human pathogens such as *Streptococcus* sp. (Lee et al., 2013), *E. coli*, *Vibrio cholera*, and *P. aeruginosa* (Marudhupandi & Kumar, 2013) has also been reported. Overall, the algal extract obtained in this work exhibited selective promotion of bacterial growth towards probiotic bacteria versus pathogenic bacteria, especially when compared to commercial FOS.



**Fig. 1.** Growth rate (%) of a selection of beneficial bacteria (*L. reuteri*, *L. plantarum*, *B. angulatum*, *L. gallinarum*, *L. casei*, *L. acidophilus* and *L. fermentum*) and pathogenic bacteria (*E. coli* and *P. aeruginosa*) induced by the algal extract from *Ascophyllum nodosum* at 0.1% (w/v) compared to other commercial carbon sources with prebiotic effect (FOS and GOS) at the same concentration. Data are expressed as mean ± SD (n = 3).

### 3.4. Rheological properties

Fig. 3A shows the viscosity of the extract dispersions in water as a function of the concentration. These exhibited a Newtonian behaviour, so their viscosities were calculated as the slope of the stress vs. strain curves. Solutions of a commercial alginate sample were also analysed for comparative purposes, and these exhibited high viscosities even at low concentrations, which increased exponentially as previously reported (Chan et al., 2009; Davarci et al., 2017). In fact, alginate concentrations used to produce hydrogel capsules are usually limited due to their high viscosity. In contrast, the viscosity of the algal extract dispersions was comparably low even at high solids concentrations, due to its relatively low alginate content (cf. Table 1). Accordingly, in order to achieve a viscosity equivalent to that of a 2% w/v commercial alginate solution, the extract suspensions had to be prepared at a concentration of 50% w/v.

The viscosity of alginate solutions has been previously reported to be higher at pH values close to neutral rather than at acidic pH (Yang et al., 2009). The carboxylic groups of alginates protonate at low pH, causing the polymeric chains to interact through hydrogen bonds and eventually precipitate (Rayment et al., 2009). The pH of the initial extract dispersion in water (at 10% w/v concentration) was slightly acidic at 4.7 ± 0.1. In order to ascertain whether an increase in the pH would positively affect the solubility of alginate in the extract, the viscosity of the extract suspensions was also studied as a function of the pH. The results are shown in Fig. 2B, and revealed that the viscosity of the extract suspensions indeed increased with the pH up to neutral pH. The pKa of uronic acid

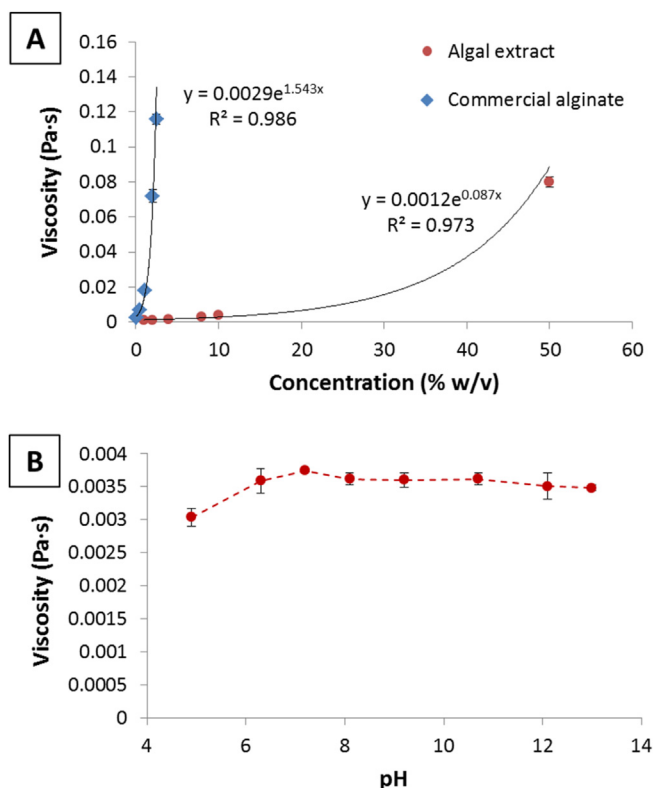


Fig. 2. Variation of the viscosity of extract suspensions and commercial alginate solutions with the concentration (A) and variation of the viscosity of 10% w/v extract suspensions with the pH (B).

residues is in the range between 3.38 (mannuronic acid) and 3.65 (guluronic acid) (Gómez-Mascaraque et al., 2019), so the viscosity of pure alginate would not be expected to continue increasing above pH 5. The different behaviour observed for the extract was related to the complexity of its composition, in which the presence of other components and/or their interactions with alginate also played a relevant role. Nevertheless, the observed increase in viscosity was relatively small, due to its low alginate content.

### 3.5. Gelling capacity of the extract

Alginate concentrations used to produce hydrogel beads through external gelation typically range between 1 and 2% (Li et al., 2016; Ramos et al.; Zhang et al., 2017), since higher concentrations yield too viscous solutions. As the alginate present in the algal extract accounted only for a small fraction of its total solids content (cf. Table 1), a concentration of 10% w/v extract was initially selected. On the other hand, concentrations of  $\text{CaCl}_2$  of 0.05–0.1 M are generally used to gel alginates (Jung et al., 2016; Lopez-Sanchez, Fredriksson, Larsson, Altskär, & Ström; Ozseker and Akkaya, 2016), so a slightly higher concentration of 0.2 M was initially selected to gel the algal extract. Hence, a 10% w/v aqueous extract dispersion was drop-wise extruded onto a 0.2 M  $\text{CaCl}_2$  bath. However, bead formation was not observed under these conditions. Therefore, different conditions were tested in order to optimize the procedure to obtain hydrogel beads from the algal extract.

#### 3.5.1. Impact of pH

The pH of the initial extract dispersion in water was  $4.7 \pm 0.1$ , i.e. slightly acidic. As commented previously, the carboxylic groups of alginates protonate at low pH (Rayment et al., 2009), preventing its dissolution due to intermolecular interactions through hydrogen bonds. Hence, it was hypothesized that increasing the pH would not only facilitate the dissolution of alginate but also the interaction between its

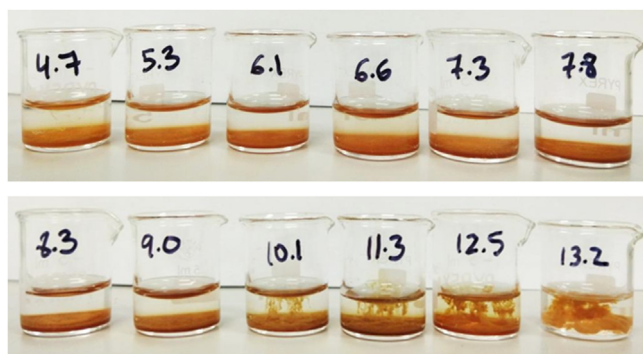


Fig. 3. Images of the algal extract after drop-wise extrusion into 0.2 M  $\text{CaCl}_2$  baths from aqueous solutions (10% w/v concentration) of increasing pHs (4.7–13.2). The pHs of the suspensions are indicated on each beaker.

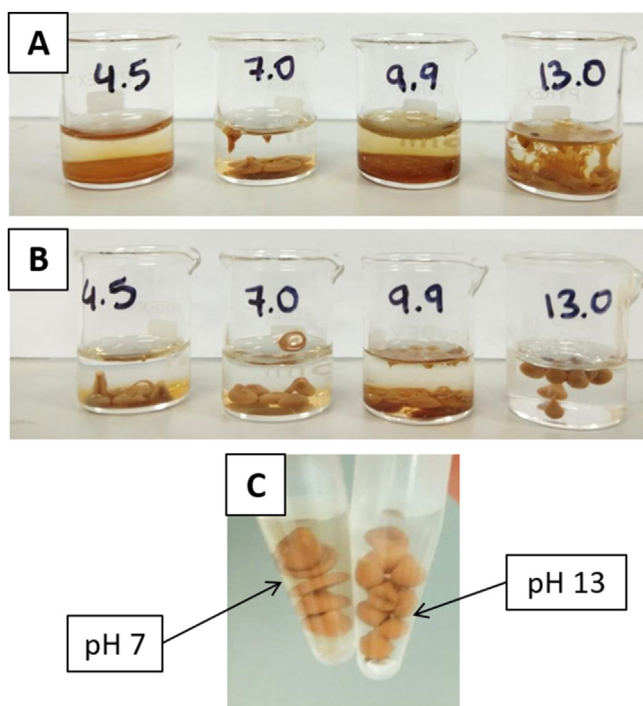
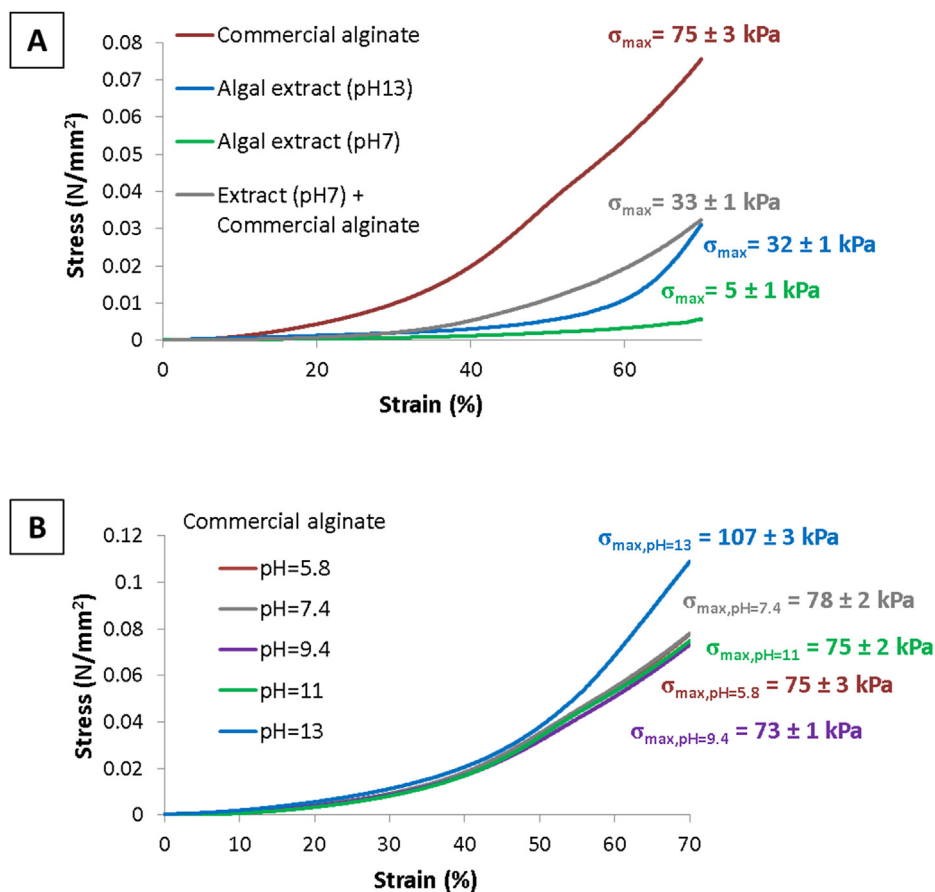


Fig. 4. Images of the algal extract after drop-wise extrusion into 0.2 M (A) and 1 M (B, C)  $\text{CaCl}_2$  baths from aqueous solutions (50% w/v concentration) of increasing pH (4.5–13.0). The pH of the suspensions are indicated on each beaker in A and B.

deprotonated carboxylic groups and the  $\text{Ca}^{2+}$  ions in the gelling solution. Accordingly, the pH of the dispersions was adjusted to increasing values and the resulting dispersions were drop-wise extruded onto 0.2 M  $\text{CaCl}_2$  baths. Fig. 3 shows the physical appearance of the resulting materials.

Indeed, the gelling ability of the extract dispersions improved with increasing pH. While at pHs below 10 the extract sedimented to the bottom of the beakers before gelling, at pHs above this value the extract droplets gelled faster upon contact with the  $\text{Ca}^{2+}$  ions. The best results were observed at a pH close to 13, for which most of the extract dispersion had gelled before reaching the bottom of the flask. However, none of the above dispersions successfully yielded spherical hydrogel beads similar to those obtained with purified alginates. This could be partly attributed to the low viscosity of the dispersions (cf. Section 3.4) and the subsequent rapid diffusion of the extract molecules to the  $\text{CaCl}_2$  bath, which turned out to be faster than the gelling of its alginate chains.



**Fig. 5.** Compression strain-stress curves of hydrogel beads and their maximum compressive stress ( $\sigma_{\max}$ ) at 70% strain. A) Hydrogel beads with different extract/commercial alginate compositions. B) Commercial alginate beads prepared at different pHs.

### 3.5.2. Impact of extract concentration

According to Davarci et al. (2017), in order to obtain spherical beads from alginate solutions, the viscosity of the droplets needs to be high enough to avoid deformations in the gelling bath. Hence, to increase the viscosity of the extract dispersions, higher concentrations of up to 50% w/v were prepared to reach a viscosity similar to that of a 2% w/v commercial alginate solution (cf. Section 3.4).

Fig. 4A shows the physical appearance of the resulting materials. Once more, the dispersion at pH = 13 was able to gel fast enough to form hydrogel structures before reaching the bottom of the gelling bath and, interestingly, at a concentration of 50% w/v, so did the sample prepared at pH = 7. It is worth noting that, although increasing the pH (up to ~7) increased the viscosity of the extract suspensions (cf. Section 3.4), samples adjusted at higher pHs were somewhat more diluted due to the addition of increasing volumes of NaOH solution to adjust the pH, which resulted in a maximum in viscosity at around pH = 7. Therefore, the best results were observed for the two extract suspensions having either the highest viscosity or the highest pH, suggesting that both factors had a relevant impact on the gelling properties of the algal extract. However, gelling of the extract under these conditions was still not fast enough to yield hydrogel beads.

### 3.5.3. Impact of $\text{Ca}^{2+}$ concentration

In order to further increase the gelling rate, the concentration of  $\text{Ca}^{2+}$  ions in the gelling bath was increased to 1 M. The results are shown in Fig. 4B, in which a much faster gelling rate was observed. Under these conditions, pseudo-spherical hydrogel beads were successfully obtained at pH = 13. Although flatter and less spherical, hydrogel beads were also obtained at pH = 7. Fig. 4C shows the appearance of the obtained beads after thoroughly washing with distilled water. The hydrogel beads

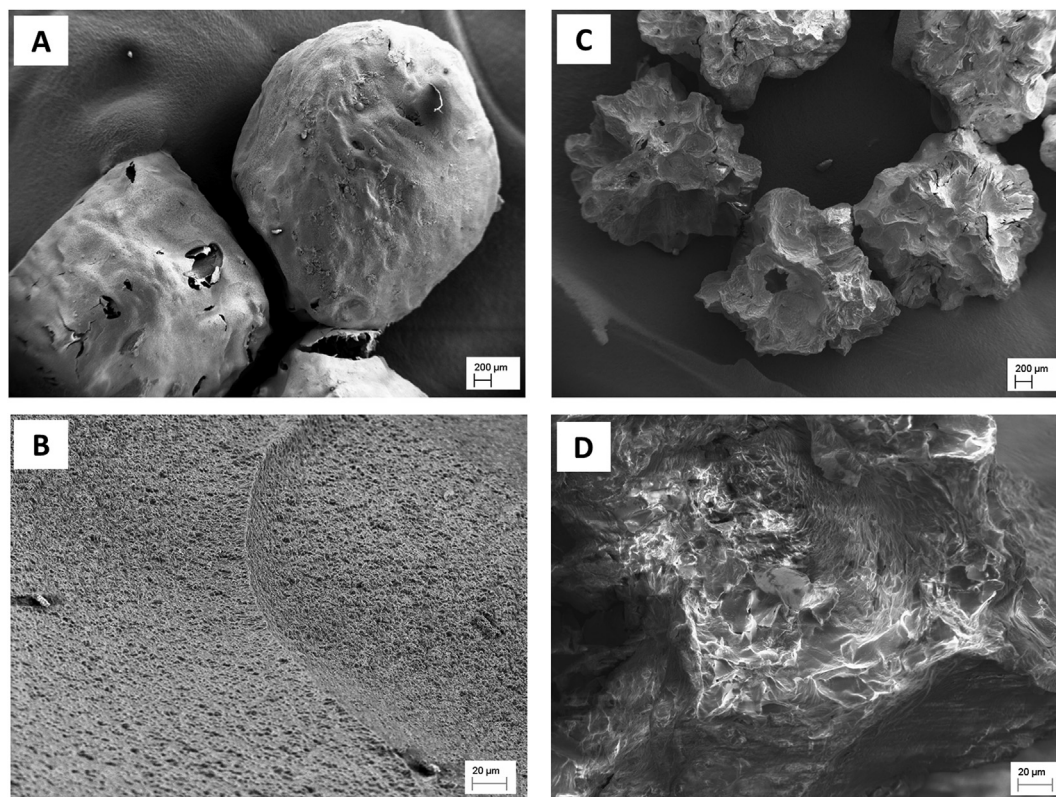
produced under these two conditions were selected for characterisation of their mechanical properties.

### 3.6. Mechanical properties of the selected hydrogel beads

Compression tests are generally accepted for the comparison of the mechanical properties or textural attributes of hydrogel samples (Ebara et al., 2014; Sharma and Bhattacharya, 2014). Thus, the selected algal extract beads (prepared both at pH = 7 and at pH = 13) were subjected to compression tests. Hydrogel beads were also produced using the commercial alginate (at a typical concentration of 1.5% w/v) and subjected to the same compression tests for comparison purposes. The obtained strain-stress curves are shown in Fig. 5A, together with the values of their maximum compressive stress at 70% strain. Complementary nanostructural analysis of these materials by SAXS can be found in the Supplementary Material.

Strain-stress curves were not linear, as is generally the case for polysaccharide-based hydrogels, due to a contribution from plastic deformation (Sharma and Bhattacharya, 2014). As expected, the algal extract beads were less stiff (i.e. offered less resistance to compression) than the pure alginate beads, due to the presence of a substantial amount of other components which did not participate in the network formation and impeded to some extent the interaction between alginate chains. This behaviour had been previously observed for gels prepared from other algal-derived extracts. For instance, higher stress values were reported for hydrogels obtained from purified agars than for those prepared from non-purified agar fractions (Martínez-Sanz et al., 2019), which was attributed to the high content of non-gelling components in the latter.

On the other hand, extract beads prepared at pH = 13 were significantly stiffer than those prepared at pH = 7, suggesting as described



**Fig. 6.** SEM micrographs of the selected extract/alginate freeze-dried capsules (A,B) and control alginate capsules (C,D) obtained at 50x (A,C) and 1000x (B,D) magnification. Scale bars correspond to 200  $\mu\text{m}$  in A and C, and 20  $\mu\text{m}$  in B and D, respectively.

above that the gelling properties of the extract were enhanced at higher pH. To investigate this further, hydrogel beads were prepared from pure commercial alginate solutions (1.5% w/v) after pH adjustment (from 5.8 to 13). Results showed that alginate beads prepared at pH = 13 were also stiffer (Fig. 5B) than those prepared at lower pH, an effect similar to that observed for the extract-based beads.

Although increasing the pH significantly enhanced the gelling properties of the algal extract, mild pHs are preferred for many applications, since excessively high pH may be detrimental for most bioactive compounds, including polyphenols (Gómez-Mascaraque et al., 2015, 2017). Therefore, the capability of the extract of gelling at neutral pH rather than at pH = 13 was considered of more practical interest. In order to enhance the mechanical properties of the beads prepared at pH = 7, a plausible strategy would be to enrich the algal extract in alginate by adding a mild purification step after extraction. As a proof of concept, this was attempted by mixing the extract suspension (50% w/v, pH = 7) with a commercial alginate solution (1.5% w/v) at a volumetric ratio of 50:50, prior to preparation of hydrogel beads (1 M  $\text{CaCl}_2$ ). These beads were also subjected to compression tests and their strain-stress curves are also shown in Fig. 5A. The results demonstrated that enriching the algal extract with small amounts of alginate enhanced their mechanical properties. The samples prepared at pH = 7 containing commercial alginate resulted in similar maximum compressive stress (at 70% strain) than those prepared at pH = 13 in the absence of alginate, and with higher compressive stress values at strains below 70%.

### 3.7. Impact of drying on the microstructure of the beads

For many applications, hydrogel beads must be dried in order to extend their shelf-life (Kwak, 2014) and to broaden their potential applications in foods or supplements. Thus, the previous formulations (i.e. pure extract beads prepared at both pH = 7 and 13, pure commercial alginate beads, and blended extract/alginate beads) were freeze-dried.

After freeze-drying, the algal extract beads were found to be extremely brittle, losing their integrity and breaking into powders upon handling (cf. Fig. S10 of the Supplementary Material). Insufficient alginate in the unpurified extract and the presence of non-gelling components which interfered in network formation caused the extremely low mechanical resistance of the dried beads.

On the other hand, both the pure commercial alginate beads and the mixed extract/alginate system led to easy-to-handle dry beads after freeze-drying. As expected, commercial alginate dry beads exhibited a shrunken, highly porous structure (Fig. 6). It is well-known that calcium alginate hydrogel beads lead to very porous structures with distorted shapes upon freeze-drying (Chan et al., 2011), due to their high water content which leaves void volumes when sublimated (Sriamornsak et al., 2008). Since the alginate concentration in the solutions has to be low (due to high viscosity), low density gel networks are generally obtained (Gómez-Mascaraque, 2017). Apart from its negative impact on the visual appearance of the dry beads (Chan et al., 2011), this usually results in low entrapment efficiencies for small water soluble compounds incorporated within the polysaccharide network (Đorđević et al., 2015), as they can easily diffuse to the gelling bath due to insufficient barrier effects (Gómez-Mascaraque, 2017). One strategy to overcome these limitations is the incorporation of filler materials within the alginate matrix (López Córdoba, Deladino and Martino, 2013), which can effectively reduce shrinkage of the alginate network (Rassis et al., 2002). SEM micrographs of the dry extract/alginate beads (cf. Fig. 6A and B) shows that the non-gelling components of the algal extract acted as fillers, limiting shrinkage of the structures and reducing their porosity.

## 4. Conclusions

This study investigated the potential of a carbohydrate-rich algal extract from the edible brown seaweed (*A. nodosum*) as a gelling agent for food applications, focusing on its bioactive and structuring properties.



The presence of alginates and fucoidans in the extract was confirmed, although other carbohydrates such as mannitol and laminarin were also present in greater amounts, due to the simplicity of the extraction protocol and the selected harvesting season. The extract also had significant amounts of phenolic compounds that were partially responsible for their bioactive properties. The algal extract was antioxidant and exhibited prebiotic potential by promoting the growth of beneficial *Lactobacillus* sp. and *Bifidobacteria* sp., but not pathogenic bacteria, when compared with a commercial prebiotic (FOS). However, solutions of the raw extract in water exhibited relatively poor gelling properties. These were considerably improved by optimising the processing conditions (i.e. pH, extract concentration and concentration of CaCl<sub>2</sub> in the gelling bath). By extruding aqueous solutions of the extract at high concentration (50% w/v), into 1 M CaCl<sub>2</sub> solutions, hydrogel beads were successfully obtained at neutral to alkaline pHs. However, the requirement of using such a high extract concentration would be a limitation to its practical use.

The mechanical properties of the beads produced at neutral pH were further enhanced by blending the extract with small amounts of commercial alginate, which suggested that including a mild purification step to enrich the quantity of alginate, or the application of novel extraction technologies to improve the alginate extraction yield, would improve its gelling properties considerably. After freeze-drying, the beads obtained from extract/alginate blends, resulted in a lower extent of shrinkage and reduced porosity compared to beads prepared from pure commercial alginate.

Overall, the carbohydrate-rich algal extract prepared in this work by simple aqueous extraction showed a comprehensive set of biological and functional properties, such as antioxidant, antimicrobial and prebiotic properties, although its gelling properties were poor due to its low alginate content. By comparing an unpurified extract from *A. nodosum* with a purified alginate, this work also sets the basis for understanding the behaviour of other extracts from *A. nodosum* with intermediate alginate contents and behaviour.

#### CRedit authorship contribution statement

**Laura G. Gómez-Mascaraque:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Project administration, Visualization, Writing – original draft. **Marta Martínez-Sanz:** Methodology, Software, Data curation, Formal analysis, Investigation, Funding acquisition, Visualization, Writing – original draft. **Rosalía Martínez-López:** Methodology, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Antonio Martínez-Abad:** Methodology, Data curation, Formal analysis, Investigation. **Bhavya Panikuttira:** Investigation. **Amparo López-Rubio:** Resources, Supervision, Funding acquisition, Writing – review & editing. **Maria G. Tuohy:** Project administration, Resources, Supervision, Funding acquisition, Writing – review & editing. **Sean A. Hogan:** Project administration, Supervision, Funding acquisition, Writing – review & editing. **André Brodkorb:** Conceptualization, Project administration, Resources, Supervision, Funding acquisition, Writing – review & editing.

#### 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.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2021.05.005>.

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