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Enzymatic approach for the extraction of bioactive fractions from red, green and brown seaweeds

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ABSTRACT

Although several enzymatic approaches have been applied with this intent, the sequential use of enzymes covering both cellulolytic and proteolytic activity has never been performed in seaweeds. Sequential use of these enzymes improved the overall extraction yield by up to 160%, 30% and 80% in the different seaweeds when compared to the control condition (water extraction), use of a carbohydrases' cocktail alone and use of proteases alone, respectively. Regarding the proximate composition of extracts, it proved to be an efficient approach for the solubilization of carbohydrates (up to 28% in *G. vermiculophylla*, 66% in *P. dioica*, 77% in *U. rigida* and 35% in *F. vesiculosus*) and protein (up to 55% in *G. vermiculophylla*, 47% in *P. dioica*, 52% in *U. rigida* and 42% in *F. vesiculosus*). For all biomasses, the combination of enzymes induced a significant increase in antioxidant activity, not only by the increase of phenolic compounds but also by the hydrolysis of protein to peptides. Moreover, extracts from red seaweeds displayed prebiotic activity which can be ascribed to their increased content in oligosaccharides and protein/peptides. Overall, the sequential use of enzymes with different activities demonstrated to be an efficient approach for extracting functional fractions to be used as functional ingredients to improve the nutritional value (e.g. in proteins) and/or to include antioxidant and prebiotic features in the food product.

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1. Introduction

Marine macroalgae, commonly known as seaweeds, are autotrophic multicellular organisms, with a great diversity of forms

and sizes. They can be categorized into three broad taxonomic groups according to their main photosynthetic pigments, namely chlorophylls, fucoxanthins and phycobilins for green algae (Chlorophyceae), brown algae (Phaeophyceae) and red algae (Rhodophyceae), respectively (Jönsson et al., 2020; Kadam et al., 2013; Mohamed et al., 2012).

In the past years, seaweed farming has been growing rapidly worldwide, being practiced in about 50 countries (FAO, 2018). However, several legal and health requirements must be taken into consideration before selecting seaweed or

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seaweed extracts for human consumption purposes. Firstly, it should be noted that only 21 seaweed or seaweed extracts are currently authorized for human consumption on the European Union, under the EU 2020/1820 and EU 2018/1023 regulations (Geada et al., 2021). Secondly, health hazards contaminants like heavy metals, pesticide residues, toxins, pharmaceuticals and the presence of pathogenic micro-organisms should be according to specific regulations (Banach, 2020). Further, the food grade should be maintained throughout the whole chain, which is particularly relevant when considering waste-streams as algae growth medium.

The growing awareness of food as a source of functional ingredients for health promotion has aroused the food industry's interest in seaweed. They are recognized as an excellent food source for health promotion, as they are rich in dietary fibres, polysaccharides, proteins/amino acids, polyunsaturated fatty acids (PUFAs), vitamins polyphenols and pigments (Charoensiddhi et al., 2017a; Paiva et al., 2017). Though seaweeds could be used for direct consumption, the industry is focused on their processing to obtain added-value products with a wider range of potential applications (food, pharmaceuticals, nutraceuticals and hydrocolloid industries). Nevertheless, the bioavailability and bioaccessibility of some nutrients is often impaired by the complex seaweed structure and composition, and mild processing or extraction may improve its nutritional value. Despite the recognized nutritional value and their enormous potential in a wide range of industries, one of the major drawbacks to their use is the lack of protocols specially tailored to the isolation of compounds or fractions of interest from this biomass (Matos et al., 2021).

Seaweed cell walls are made up of rigid and chemically complex and heterogeneous biomolecules i.e., sulfated and branched polysaccharides associated with proteins and several bound ions that limit the extraction of bio compounds (Rodrigues et al., 2015). Traditionally, the extraction of interest compounds in seaweed is water-based. This extraction has the advantage of being inexpensive, environmentally friendly and food compatible, however, it has low extraction efficiency and selectivity (Matos et al., 2021). Extractions with organic solvents such as diethyl ether, benzene and acetonitrile have higher yields and selectivity, however, these methods are non-food compatible and/or harmful to the environment (Rodrigues et al., 2015; Wijesinghe and Jeon, 2012).

The application of cell wall degrading food-grade enzymes such as carbohydrases and proteases to seaweeds has been growing considerable interest in the scientific community due to their hydrolytic action. It has the advantage of being an eco-friendly and non-toxic technology since it alleviates the use of solvents in the process (Charoensiddhi et al., 2017a; Kadam et al., 2013; Ummat et al., 2021). Enzyme-assisted extraction is a high bioactive yielding technology where the rigid and heterogeneous cell wall structures are weakened or disrupted, releasing the interest biocompounds to the extraction medium. In addition, it also removes the barriers to water solubility, thus reducing the insolubility of bioactive compounds and preserving their biological value (Kadam et al., 2013; Wijesinghe and Jeon, 2012).

Considering the limitations of water-based extraction and aiming to increase the extraction efficiency of target compounds using food-compatible methods, the main objective of this work was to study the effect of a multi-enzyme complex containing a wide range of carbohydrases (Viscozyme® L) and two proteases (Flavourzyme® and

papain), alone or in combination, in green (*Ulva rigida*), brown (*Fucus vesiculosus*) and red seaweeds (*Gracilaria vermiculophylla* and *Porphyra dioica*). Despite the several reports of single enzyme approaches used in seaweeds, to the best of the authors' knowledge, the extensive characterization and evaluation of the synergetic effect of enzymes in these bio-masses is a novelty. To achieve this goal, the chemical characterization of the different seaweeds was performed, alongside the optimization of the extraction conditions using the different enzymes. In addition, the chemical characterization of the extracts and the evaluation of their antioxidant and prebiotic potential were also assessed.

2. Materials and methods

2.1. Raw material

The seaweeds used in this study were supplied dried by AlgaPlus (Ílhavo, Portugal). *G. vermiculophylla* (batch G1.01119. D: B1) was washed twice with tap water and once with distilled water to remove all the debris. Subsequently, they were dried at 60 °C for 16 h and stored in the dark at room temperature until use. Samples of *P. dioica* (batch P1.01019.M.B1), *U. rigida* (batch U1.00219. M) and *F. vesiculosus* (batch F1.00719.M.B1) were purchased ready to use (flakes < 10 mm). The same batch of all seaweeds (submitted to the same standardized procedures at AlgaPlus) was used in all tests to avoid differences in the initial biomass so that the results could be ascribed to the different treatments used.

2.2. Chemical characterization of raw material

Each seaweed was analyzed for ashes, carbohydrates, lipids, and proteins following standard protocols. The ash content (NREL/TP-510-42618) was determined by calcination in a muffle furnace at 575 °C until achieving a constant weight (Sluiter et al., 2008a). The nitrogen content of the biomass samples was measured by Kjeldahl digestion (NREL/TP-510-4262) (Hames et al., 2008) and the protein content was estimated using a nitrogen factor of 5 (Angell et al., 2016). The lipid content was determined by Bligh and Dyer method with chloroform and methanol (Bligh and Dyer, 1959).

To assess the carbohydrate content sequential Soxhlet extractions with (i) distilled water for 16 h and (ii) 80% v/v ethanol for 8 h were carried out to remove water-soluble and liposoluble compounds, respectively (Sluiter et al., 2012, 2008b). For oligosaccharide quantification, aliquots from the extractions and residues were subjected to hydrolysis with 4% H₂SO₄ (121 °C, 20 min). The resulting post-hydrolyzed liquid and the directly extracted liquid were filtered through 0.22 µm membranes and analyzed by high-performance liquid chromatography (HPLC) to quantify the solubilized compounds such as uronic acids (i.e., glucuronic, galacturonic and manuronic acids were used as standards) and sugars (i.e., glucose, galactose, xylose, mannose, arabinose, rhamnose, fucose and mannitol were used as standards). The conditions used in the HPLC analysis were as follows: refractive index detector; UV detector at 210 nm; Aminex HPX-87H column at 60 °C; and a mobile phase of 0.05 mol/L H₂SO₄ at a flow rate of 0.6 mL/min. Extract-free seaweed was subjected to a two-step quantitative acid hydrolysis (QAH) with: (i) 72% H₂SO₄, 60 min, 30 °C and (ii) 4% H₂SO₄, 60 min, 121 °C. The liquid from the QAH was also analyzed by HPLC, using the same conditions as before. Uronic acids, glucan,

galactan, xylan, mannan, arabinan, rhamnan, fucoidan and mannitol groups were calculated from the concentrations of the respective monomeric sugars, using the standardized correction factor of 132/150 for pentoses and 162/180 for hexoses. The insoluble phase from the QAH was subjected to gravimetric quantitation in number 3 Gooch crucibles and reported as an acid-insoluble residue (AIR).

2.3. Optimization of enzyme-assisted extraction

The optimization of **enzyme-assisted extractions** was performed on *G. vermiculophylla*. The pH (4.5 for Viscozyme® L and 7.0 for papain, Flavourzyme®) and temperature (50 °C) conditions were chosen according to the manufacturer's specifications.

Before the **enzyme-assisted extractions**, the cellulolytic and proteolytic activity of the enzymes was determined in filter paper units (FPU) (Miller, 1959) and tyrosine release from casein (Murado et al., 2009), respectively. The extraction conditions for *G. vermiculophylla* were optimized considering two steps: a pre-treatment with Viscozyme (Visc) and the treatment with Flavourzyme (Visc_Flav) or papain (Visc_Pap).

The first experimental design (pre-treatment with Viscozyme) was applied to test the effects of the independent variables, time (X_1) (7.9–28.1 h), cellulolytic enzyme concentration (X_2) (3.1–61.9 FPU/g of seaweed) and solid:solvent ratio (weight:volume; w-v) (X_3) (1–11% w-v).

The optimization of the subsequent treatment with proteases was made by testing the effects of the independent variables, time (X_1) (1.2–6.8 h) and proteolytic enzyme concentration (X_2) (1378.7–5621.3 U/g of seaweed), considering the optimal conditions for the pre-treatment and adjusting the media to the optimal pH of the selected proteases. Results were evaluated through a 2^2 central composite design with four replicates at the central point. The extraction yield (g of extract / g of seaweed \times 100) was taken as the dependent variable for both experimental designs. The correlation between dependent (Y_n) and independent variables (X_1 , X_2 and X_3) for the cellulolytic enzyme and (X_1 and X_2) for the proteolytic enzyme was established by the empirical models, considering the linear, quadratic and interaction effects of all studied variables. The regression coefficients were calculated from the experimental data by multiple regression using the least-squares method.

2.4. Recovery of biocompounds using enzyme-assisted extraction

In this study, **enzyme-assisted extractions** were performed on four different seaweeds such as *G. vermiculophylla* and *P. dioica* (red seaweeds), *U. rigida* (green seaweed) and *F. vesiculosus* (brown seaweed). The optimal conditions for each step obtained in section 2.3 were then applied to all seaweeds, considering different enzyme combinations, and performed in triplicate for each combination. After the extractions, the enzymatic reactions were stopped by heating the sample at 90 °C for 10 min followed by immediate cooling in an ice bath. The enzymatic aqueous solutions were then centrifuged at 5000 rpm for 20 min and the supernatant was kept at – 20°C until analysis.

2.5. Chemical characterization of the extracts

Total phenolic content (TPC) was determined by the colorimetric method of Folin–Ciocalteu according to Singleton and Rossi, (1965) and optimized to 96-well plates (Teixeira-Guedes et al., 2019). Results were expressed as g of GA equivalent per 100 gram of seaweed extract. Total flavonoid content (TFC) was assessed by the aluminium chloride complexation method according to Domínguez-Perles et al., (2014) with some modifications (Teixeira-Guedes et al., 2019). Results were expressed as g of catechin equivalent per 100 gs of seaweed extract.

2.6. Determination of the antioxidant activity

The antioxidant activity of the different extracts was determined using three different methods, ABTS (Domínguez-Perles et al., 2014) and DPPH (Blois, 1958) and ferric reducing antioxidant power (FRAP) (Bolanos de la Torre et al., 2015) as previously optimized by Teixeira-Guedes et al., (2019). Oxygen radical absorbance capacity (ORAC) assay was applied to extracts used for the evaluation of prebiotic potential. ORAC procedure was performed according to the method described by Coscueta et al. (2020). The antioxidant activity of the samples was determined by interpolation of the standard curve for Trolox. In the ABTS and DPPH radical scavenging methods, the % of radical inhibition was calculated using the following Eq:

$$\%inhibition = \frac{Abs_{Radical} - Abs_{Sample}}{Abs_{Radical}} \times 100 \quad (1)$$

2.7. Evaluation of proteins and peptides profile by size exclusion chromatography

The molecular weight distribution was determined using the AKTA Pure 25 system (GE Healthcare Life Sciences, Freiburg, Germany) with a configuration of two pumps, a gel filtration column prepacked with Superdex 200 10/300 GL, a column Superdex Peptide 10/300 GL and an UV multiwavelength detection monitor U9-L. The column was operated at a flow rate of 0.5 mL/min with 0.025 M phosphate buffer (pH 7) with 0.15 M NaCl and 0.2 g/L NaN₃. The absorbance was monitored at 280 nm. Standard proteins with known molecular weights were used to establish the molecular weight standard curve (Thyroglobulin, 669 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) (Campos et al., 2019) and the antihypertensive peptide KGYGGVSLPEW (1.2 kDa).

2.8. In vitro simulation of gastrointestinal digestion

In vitro simulation of the gastrointestinal (GI) tract was performed in an extract from *G. vermiculophylla* and *P. dioica* subjected to pre-treatment with Viscozyme and treatment with Flavourzyme. For each replicate, 1 g of each extract was dissolved in 20 mL of phosphate buffer. The system used aimed to simulate the conditions of each phase of the GI tract (mouth, stomach and small intestine) (Campos et al., 2020). For the mouth simulation, samples were mixed with simulated oral fluid, the pH was adjusted between 5.6 and 6.9 and α -amylase enzyme (100 U/mL) was added at a rate of 0.6 mL/min. Samples were incubated for 2 min at 37 °C, at 200 rpm.

In the gastric simulation, simulated gastric fluid was added and pH was adjusted to 2.0. The enzyme pepsin (25 mg/mL) was added at a rate of 0.05 mL/mL of sample. Digestion was performed for 2 h at 37 °C, 130 rpm. In the last step, a small intestine simulation was performed by adding intestinal gastric fluid and adjusting the pH to 6.0 (NaHCO₃, 1 M). A mixture of pancreatin enzyme (2 g/L) and bile salts (12 g/L) was added at a rate of 0.25 mL/mL of sample. Intestinal digestion took place for 120 min at 37 °C, at 45 rpm.

2.9. Prebiotic potential

The prebiotic potential was tested in digested and undigested extracts of *G. vermiculophylla* and *P. dioica* at a final concentration of 1% and 2% according to Bordiga et al (2019). The prebiotic strains used were *Lactobacillus acidophilus* Ki (LAS) (isolated from fermented milk, CSK, Netherlands), and *Lactobacillus casei* (*L. casei*) L26 (DSM - Moorebank, NSW, Australia), *Bifidobacterium animalis* spp. *lactis* Bb12 (Chr. Hansen, Denmark) and *Bifidobacterium animalis* Bo (CSK, Ede, Netherlands). Each culture was propagated twice in the appropriate medium at 37 °C for 24 h. The growth of *Lactobacillus* was performed using Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France), while *Bifidobacterium* was grown in MRS broth supplemented with 0.5 g/L of L-cysteine-HCl (Fluka, Switzerland). Prior to the experiment, the bacteria were grown for 24 h at 37 °C in the mentioned mediums. The reaction was performed in 96-well-microplates.

Tested samples were essentially rich in carbohydrates and proteins/peptides. Thus, to understand how extracts affect bacterial growth, two tests were performed for each sample. In the first test, bacterial strains were grown in media with 2% glucose and in the second test in media without a sugar source. All assays were performed in triplicate. For each test, 2% of bacterial inoculum was used. Fructooligosaccharide (FOS) (Orafti P 95®; Orafti, Belgium) (2%) was used as the positive control. Wells with *Bifidobacterium* were sealed with paraffin to assure anaerobiosis conditions. The microplate was incubated for 48 h at 37 °C in a microplate reader and bacterial growth was monitored at 660 nm, by measuring the optical density (OD) every hour.

2.10. Statistical analysis

Results were presented as mean ± standard deviation (SD) of three independent experiments. Statistical analyses were performed using Graph Pad Prism 6 (GraphPad Software, Inc., San Diego, CA). Differences between samples were tested using analysis of variance (ANOVA) followed homogeneity test and Tukey's multiple comparisons test. Results were considered as being statistically significant for values of $p < 0.05$.

3. Results and discussion

3.1. Composition of raw material

Seaweeds have an exceptional combination of macro- and micronutrients that make them very interesting from a nutritional perspective. However, the concentrations of such elements are very susceptible to seasonality, environmental conditions, geographical origin and several other factors making generalizations of algal composition very difficult (Sharma et al., 2018). Since the chemical composition of the seaweed, such as the monomers profile, differs in nutritional value, fermentability, and biological and functional behaviour leading to different industrial applications, such as fermentation medium, biodiesel, texturizing agents, natural pigments, proteins sources, the authors considered this step to be of the foremost importance. Thus, the chemical composition of each seaweed is presented in Table 1. The same batch of each seaweed was used through all the work to allow direct comparisons between different treatments.

Higher carbohydrate content was found in *G. vermiculophylla* followed by *F. vesiculosus*, *P. dioica* and *U. rigida*. Regarding carbohydrate profiles, as can be observed in Table 1, values found in the present study are in concordance with the available literature.

The major storage carbohydrates and cell polymers are common in this taxonomic group: red agarophyte seaweeds are composed of glucose-based reserve storage floridean, cellulose, mannan and agar composed by galactopyranose and galactose. In the case of *P. dicoica*, the main polysaccharide is an agarose-related constituent porphyran, composed of galactosyl and 3,6-anhydro- α -L-galactosyl units,

Table 1 – Chemical characterization of raw material.

Component g/100 g DW	<i>G. vermiculophylla</i>	<i>P. dioica</i>	<i>U. rigida</i>	<i>F. vesiculosus</i>
Ash	28.3 ± 0.18 ^c	20.8 ± 0.55 ^a	35.3 ± 0.13 ^d	25.9 ± 0.91 ^b
Crude Protein	15.9 ± 0.07 ^c	26.7 ± 0.07 ^d	14.8 ± 0.13 ^b	9.24 ± 0.16 ^a
Crude Lipid	1.24 ± 0.06 ^a	2.00 ± 0.14 ^b	3.52 ± 0.34 ^c	7.90 ± 0.54 ^d
Water extractives	32.5 ± 4.20 ^a	31.8 ± 0.42 ^a	37.0 ± 0.86 ^a	35.6 ± 1.42 ^a
Ethanol extractives	3.74 ± 0.40 ^a	5.86 ± 0.33 ^b	4.23 ± 0.11 ^a	7.28 ± 0.14 ^c
Carbohydrates	39.1 ± 2.02 ^c	28.8 ± 0.48 ^b	21.8 ± 1.22 ^a	36.5 ± 1.01 ^c
% Uronic acids	5.0 ± 0.03 ^a	5.06 ± 0.47 ^a	15.0 ± 0.27 ^b	30.2 ± 0.25 ^c
% Glucose units	15.7 ± 0.67 ^c	3.30 ± 0.70 ^a	43.9 ± 0.58 ^d	13.9 ± 0.25 ^b
% XMG units	67.2 ± 0.77 ^b	75.7 ± 0.72 ^c	9.17 ± 0.24 ^a	9.23 ± 0.60 ^a
% Rhamnose units	-	-	32.0 ± 0.52	-
% Arabinose units	12.1 ± 0.13 ^b	15.93 ± 0.08 ^c	-	0.33 ± 0.05 ^a
% Fucose units	-	-	-	23.7 ± 0.34
% Mannitol units	-	-	-	22.6 ± 0.38
AIR	0.92 ± 0.15 ^a	0.92 ± 0.17 ^a	11.7 ± 0.22 ^b	14.7 ± 0.83 ^c

DW – dry weight; XMG - xylose, mannose, galactose; AIR – acid insoluble residues

with minor 3,6-anhydrogalactosyl residues and partial methylation. On the other hand, green seaweeds' cell walls are composed of cellulose and xyloglucans, while their sulfated polysaccharides are composed by uronic acid-rich polysaccharides containing rhamnose, xylose and galactose residues. Brown seaweeds are typically composed by laminaran, mannitol, alginate/alginic acids, that are composed of mannuronic and guluronic acid units and fucoidans, a sulfated fucose-rich polysaccharides that can present galactose, mannose, xylose, glucose, glucuronic acid residues (Stiger-Pouvreau et al., 2016).

Although only about 80% of the seaweed composition was identified, it is predicted that the remaining fraction is constituted by carbohydrates that, due to their particular structure, are known to react poorly to the two-step acid hydrolysis commonly used for the determination of structural carbohydrates in biomass, namely uronic acids (Manns et al., 2014), mannitol and fucose (Kostas et al., 2016) and 3,6-anhydro- α -galactose (Park et al., 2012). However, phenolic compounds and pigments were also present but in small quantities (Peñalver et al., 2020).

Protein determination in seaweeds presents a very high uncertainty associated with the conversion factor, some authors use the standard nitrogen conversion factor of 6.25 for all seaweeds, while others refer to factors for specific groups or even species. Based on the lack of consensus, the authors have chosen to use the conversion factor of 5 proposed by Angell et al. (2016), as the most accurate universal seaweed nitrogen-to-protein conversion factor. *P. dioica* presented higher protein content, circa 27% in dry weight when compared to *G. vermiculophylla* (16%), *U. rigida* (15%) and *F. vesiculosus* (9%). Values found in *P. dioica* were higher than several legumes (e.g., common bean, peas chickpeas) and comparable with that of high-protein plant foods such as soybean and lupins (Rizzo and Baroni, 2018), thus, representing an excellent food source of dietary protein.

The values of ash observed in this study ranged from 20% to 35% in the different seaweeds, *U. rigida* presenting the highest values. In all seaweeds, lipids values were low, representing below 8% of dry matter. For all seaweeds, ash, lipids and proteins are within the reported ranges for all seaweeds (del Río et al., 2020; Mæhre et al., 2014; Neto et al., 2018; Silva et al., 2015; Varela-Álvarez et al., 2019).

3.2. Optimization of enzymatic hydrolysis conditions

In this work, it was considered the sequential use of two different types of enzymes. The multi-enzyme complex Viscozyme contains a wide range of carbohydrase activities, including β -glucanase, arabinase, cellulase, hemicellulase and xylanase to exert a broader action on the heterogenic cell wall of different seaweeds, as a preliminary structure disruption step or pre-treatment. The proteolytic enzymes Flavourzyme and papain, both with endopeptidase and exopeptidase activity, were used to help further rupture the algal cell wall and release internal storage material. This may enhance the recovery of the usually inaccessible intracellular compounds, additionally increasing the biological activities of the extract, namely, the antioxidant activity. In addition, it may also enhance protein solubilization, bioaccessibility and bioactivity, by breaking down and releasing soluble bioactive peptides.

Table 2 shows the optimization of the extraction condition with Viscozyme based on the extraction yield (%)

Table 2 – Conditions applied on the extraction with Viscozyme for *G. vermiculophylla*.

Run	Time X ₁ (h)	Viscozyme X ₂ (FPU/g)	Solid:solvent ratio X ₃ (%)	Yield Y ₁ (%)
5	12.0 (–1.00)	15.0 (–1.00)	9.00 (1.00)	19.59
14	18.0 (0.00)	61.9 (1.41)	6.00 (0.00)	36.50
15	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	34.01
2	12.0 (–1.00)	50.0 (1.00)	3.00 (–1.00)	34.85
5	12.0 (–1.00)	50.0 (1.00)	9.00 (1.00)	22.41
18	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	35.49
1	12.0 (–1.00)	15.0 (–1.00)	3.00 (–1.00)	22.93
9	18.0 (0.00)	32.5 (0.00)	1.00 (–1.41)	38.20
4	24.0 (1.00)	50.0 (1.00)	3.00 (–1.00)	42.44
13	18.0 (0.00)	3.07 (–1.41)	6.00 (0.00)	17.61
3	24.0 (1.00)	15.0 (–1.00)	3.00 (–1.00)	30.54
11	7.9 (–1.41)	32.5 (0.00)	6.00 (0.00)	18.82
17	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	34.96
8	24.0 (1.00)	50.0 (1.00)	9.00 (1.00)	38.36
16	18.0 (0.00)	32.5 (0.00)	6.00 (0.00)	35.98
7	24.0 (1.00)	15.0 (–1.00)	9.00 (1.00)	21.47
10	18.0 (0.00)	32.5 (0.00)	11.1 (1.41)	19.52
12	28.1 (1.41)	32.5 (0.00)	6.00 (0.00)	33.80

obtained in *G. vermiculophylla* seaweed. The subsequent optimization with Flavourzyme or papain in *G. vermiculophylla* was performed in carbohydrase pretreated seaweed and is presented in Table 3. Based on the fitting parameters listed in Table 4, it can be observed that the dependent variables were well interpreted by the empirical model, as can be noted by the R² and F values. The extraction yield was directly correlated with time and enzyme concentration and inversely correlated with the solid:solvent ratio. The higher extraction yield was observed after 24 h incubation with 50 FPU/g of seaweed using 3% solid:solvent (run 4). On the application of Flavourzyme, a higher extraction yield was obtained between 4 and 6 h incubation and 3500–5621 U/g of seaweed. For papain, 4–6 h incubation and 5000–5621 U/g of seaweed showed the best results. Based on these results, for the pre-treatment with Viscozyme, the conditions of 24 h incubation with 50 FPU/g of seaweed using a solid:solvent ratio of 3% were chosen. On the treatment with proteolytic enzymes, 5000 U/g of seaweed during 5 h for Flavourzyme and 6 h for papain were applied.

3.3. Extraction yield

Using the operational conditions selected, different combinations of enzymatic treatments were tested for all the seaweeds. The extraction yields achieved for each extraction condition of the four different seaweeds are presented in Fig. 1. Higher extraction yields were observed for *U. rigida* (green algae), followed by *P. dioica*, *G. vermiculophylla* (red algae) and *F. vesiculosus* (brown algae) extracts. For all seaweeds, the use of cellulolytic enzyme cocktails resulted in a significant increase (40–80%) in the overall extraction yield, while the use of proteases resulted in a smaller increase (10–40%), most of the time not significant in comparison with the control (extraction with water, at the same temperature for the same extraction time). However, when combined, cellulolytic and proteolytic enzymes proved to have a synergistic effect on the red and green seaweeds. In particular, the sequential use of a carbohydrase followed by protease showed an increase in the extraction yield of around 40–60% when compared to water extraction. Red seaweeds showed

Table 3 – Conditions applied on the extraction with Flavourzyme and Papain after pre-treatment with Viscozyme for *G. vermiculophylla*.

Run	Time X ₁ (h)	Proteolytic enzymes X ₂ (U/g)	Yield Flavourzyme Y ₂ (%)	Yield Papain Y ₃ (%)
8	6.80 (1.41)	3500 (0.00)	47.38	50.99
1	2.00 (–1.00)	2000 (–1.00)	40.48	42.21
10	4.00 (0.00)	3500 (0.00)	47.31	51.40
11	4.00 (0.00)	3500 (0.00)	47.87	51.04
2	6.00 (1.00)	2000 (–1.00)	42.68	44.48
3	2.00 (–1.00)	5000 (1.00)	42.99	43.06
7	1.20 (–1.41)	3500 (0.00)	36.93	38.25
9	4.00 (0.00)	3500 (0.00)	48.52	50.21
6	4.00 (0.00)	5621 (1.41)	48.89	51.79
4	6.00 (1.00)	5000 (1.00)	48.43	51.80
12	4.00 (0.00)	3500 (0.00)	47.56	50.55
5	4.00 (0.00)	1379 (–1.41)	40.61	42.84

Table 4 – Correlation coefficients for the selected responses and the corresponding significance level.

Run	Y ₁	Y ₂	Y ₃
a ₀	35.006	47.819 *	50.802 *
a ₁	–4.419	2.497 *	2.606 *
a ₂	–1.748	–1.485 *	–2.011 *
a ₃	4.263	2.803 *	3.983 *
a ₄	–2.649	–2.782 *	–2.860 *
a ₅	5.513	0.809	1.623
a ₆	–2.385	–	–
a ₇	0.330	–	–
a ₈	–0.516	–	–
a ₉	1.757	–	–
R ²	0.953	0.951	0.936
F	8.092	22.823	17.857

the greatest increase, approximately 40% in Visc_Pap and 75% in Visc_Flav. This phenomenon occurs due to the more intensive and/or diverse degradation of cell walls and is also linked to a different composition profile and bioactivity. This raises, once more, the need for and importance of a thorough characterization of extracted fractions. The synergistic effects have never been tested but the use of cellulolytic or proteolytic enzymes has been previously reported as having a positive effect on extraction yield (Nadar et al., 2018).

3.4. Proximate characterization of the extracts

The proximate composition of the extracted fractions is presented in Table 5. Considering the total carbohydrate yield, the combination of Viscozyme and Flavourzyme (Visc_Flav) proved to be the most efficient enzymatic approach for all four seaweeds, ranging from 25% total carbohydrates solubilization in *G. vermiculophylla* to a maximum of 78% in *U. rigida*. Simultaneously, it was observed that each seaweed's depolymerization was differently influenced by this combination of enzymes, in the red seaweed *P. dioica*, the most significant sugar solubilization is associated with carbohydrates rich in xylose/mannose/galactose units (representing 85% of total extracted carbohydrates), while *G. vermiculophylla* presents an extract composed of carbohydrates with similar amounts of glucose and xylose/mannose/galactose units (47% and 45% of total extracted carbohydrates). Due to the HPLC operational conditions used in this work, it is not possible to differentiate xylose, mannose and galactose, since these present the same retention time.

However, galactose is expected to be the majority sugar present in red seaweeds, due to their reported composition (known to be rich in agar or porphyran, with only minor traces of other monomers) (Torres et al., 2019). These ratios differ from the initial biomass composition assessed, indicating a higher affinity for the enzyme mixture towards the sulfated galactan in *P. dioica* and the structural glucan (e.g., cellulose) in *G. vermiculophylla*. These results can be a consequence of the higher molar ratio of xylose:galactose units in porphyran (1:50) than in agar (1:174), causing a higher affinity towards the carbohydrase used in this study and leading to higher hydrolysis (Pereira et al., 2021; Qiu et al., 2021). This result is of high interest since marine galactose can be efficiently converted into tagatose, a low glycemic index and low caloric value equivalent to sucrose (Baptista et al., 2021). Similar results were obtained for the uronic acids in *F. vesiculosus*, with this component corresponding to 77% of total extracted in the condition Visc_Flav. A higher amount of uronic acids in fucose-containing fractions (oligosaccharides) is associated with a higher antibacterial and antiviral effect and antioxidant activity (Ayrapetyan et al., 2021; Shen et al., 2018; Zhao et al., 2008).

Overall, the use of Viscozyme increased the extraction yield of carbohydrates and significantly changed the profile of the extracted sugars (Table 6). The most representative example of this change was the detection of arabinose and glucose units on *P. dioica* extracts only on the fractions obtained with Viscozyme. The same phenomena were observed for arabinose-rich carbohydrates' extraction on *G. vermiculophylla* and xylose-rich carbohydrates' extraction on *U. rigida*. Since these are structural components of the seaweed cell walls, it corroborates the increased hydrolysis yields and subsequent extraction efficiency obtained when enzymes are applied, being the synergistic effect more pronounced on the green seaweed (with carbohydrates richer in glucose units). Furthermore, the synergetic effect of protease and carbohydrases was more pronounced on the green seaweed (richer in glucose units), resulting in a 10-fold increase of carbohydrates extraction over control and use of proteolytic enzymes alone. Since the nutraceutical and bioactive properties of the carbohydrates depend not only on their composition but also on their size, the distinction between monomeric sugars and oligosaccharides/low molecular weight polysaccharides was assessed in all the extracts. This determination was performed based on the sugar content of each extract (measured by HPLC) before and after dilute acid hydrolysis. As can be

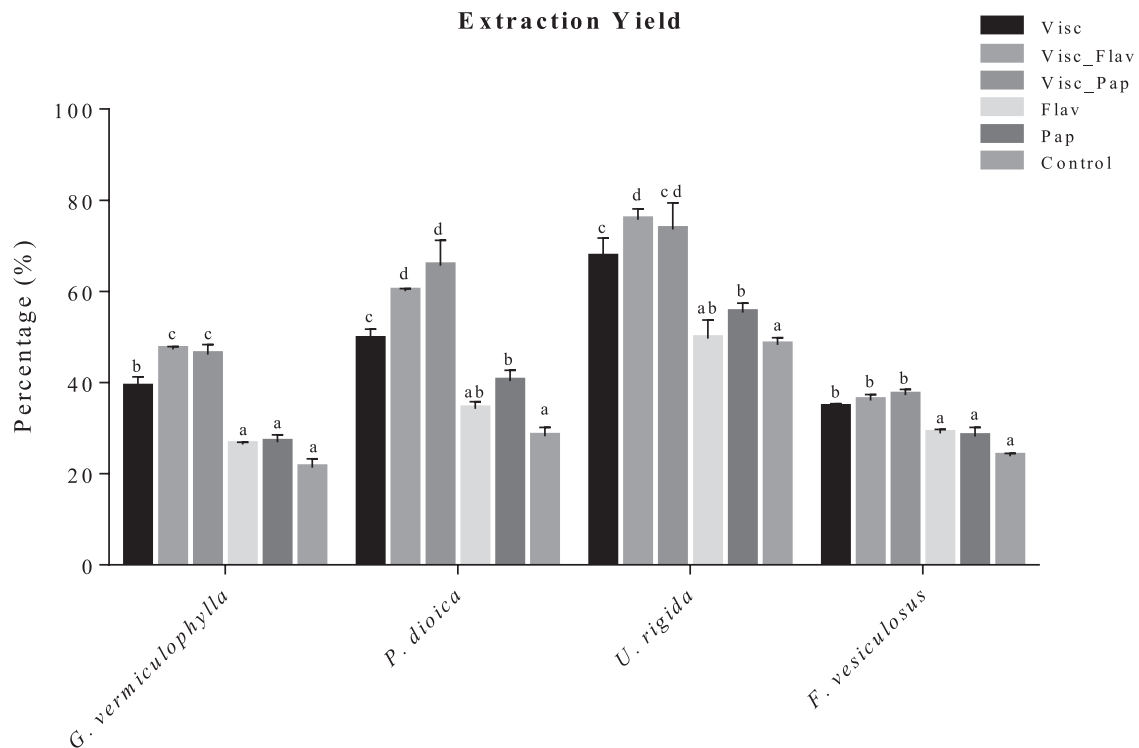


Fig. 1 – Extraction yields of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap). Different letters indicate significant differences between extracts for each seaweed at $p < 0.05$.

observed in Fig. 2, the use of Viscozyme, alone or in combination with proteases, resulted in an increased size of the carbohydrates extracted in both red seaweeds. The liquid extracts of *P. dioica* only presented arabinose oligomers and/or solubilized polysaccharides, in concentrations ranging from 19 to 25 mg per g of seaweed. Fractions from *G. vermiculophylla* presented a concentration of polymerized arabinose- and galactose-based compounds more than 15-fold greater, increasing from 10 mg per g of seaweed in non-carbohydrase assays up to 215 mg per g of seaweed when these enzymes were employed. These findings can be a result of the lack of affinity of Viscozyme for the red seaweed specific carbohydrates, aligned with their affinity for the glucose-based cell wall components. Although the lack of affinity, the use of carbohydrases led to depolymerization and weakened the cell wall. The intracellular storage polysaccharides were exposed and, despite not being subjected to complete hydrolysis, they were somewhat depolymerized and/or solubilized. The same does not apply to the remaining seaweeds, where the use of these enzymes did not change carbohydrate complexity. Since these seaweeds' carbohydrates present more glucose, xylose and rhamnose units, they are more susceptible to the enzymatic cocktail used.

Based on the knowledge that oligosaccharides and low molecular weight polysaccharides are associated with several beneficial effects (Cheong et al., 2018), the use of these sequential combination enzymes is recommended for health, cosmetic and nutraceutical applications, as it results in the formation of higher amounts of oligosaccharides.

Overall, the use of cellulolytic and proteolytic enzymes increased significantly the protein yield when compared to the control (water extraction). Enzymatic hydrolysis with Viscozyme followed by papain proved to be the most efficient approach to extract protein for all seaweeds, although both Flavourzyme and papain have endo- and exopeptidases

activity. Specifically, the combination of Viscozyme and papain resulted in a total of 55% of protein solubilization in *G. vermiculophylla*, 47% in *P. dioica*, 52% in *U. rigida*, and 42% in *F. vesiculosus*. The combination of Viscozyme followed by Flavourzyme seems to be more efficient for the extraction of carbohydrates, although the differences were not statistically significant.

Seaweeds are a rich source of phenolic compounds, of which phenolic acids, flavonoids, tannins and particularly phlorotannins are prevalent (Kadam et al., 2013). In the present work, total phenolic content (TPC) and total flavonoid content (TFC) were analyzed by colorimetric assays and are presented in Table 5. Among the seaweeds, *U. rigida* had the lowest values of TPC (0.087–0.390 g/100 g DW) when compared to *G. vermiculophylla* (0.129–0.439 g/100 g DW), *P. dioica* (0.408–0.639 g/100 g DW) and *F. vesiculosus* (0.371–0.599 g/100 g DW). Regarding TFC, the lowest values were found in both red seaweeds, *G. vermiculophylla* (0.049–0.065 g/100 g DW) and *P. dioica* (0.044–0.070 g/100 g DW).

Nonetheless, for all seaweeds, the enzyme-assisted extracts presented an increase of TPC (> 1.6-fold) and TFC (approximately 1.4-fold) when compared to the control water extract. Despite what was previously reported by Habeebullah et al., (2021), where proteases induced a higher release of TPC in comparison to carbohydrase, similar values of TPC were found in extracts obtained by Viscozyme, papain and Flavourzyme. The sequential treatment with Viscozyme and Flavourzyme and Viscozyme and papain increased significantly TPC extraction in all seaweeds in comparison with enzymes used alone. In algal cell walls, phenolic compounds are attached mainly to proteins and polysaccharide moieties (Deniaud-Bouët et al., 2014). Thus, the use of carbohydrases and proteases can break the links of phenolic compounds to proteins and polysaccharides, releasing and, consequently, increasing their extraction yield.

Table 5 – Characterization of extracts on carbohydrates, protein and total phenolic content (TPC) and total flavonoid content (TFC) in the different seaweeds.

Seaweed	Carbohydrates (g/100 g DW)	Protein (g/100 g DW)	TPC (g/100 g DW)	TFC (g/100 g DW)
<i>G. vermiculophylla</i>				
Visc	9.88 ± 0.83 ^b	5.40 ± 0.30 ^{a,b}	0.246 ± 0.018 ^{b,c}	0.046 ± 0.004 ^a
Visc_Flav	10.57 ± 0.78 ^b	7.97 ± 0.49 ^d	0.439 ± 0.034 ^d	0.059 ± 0.004 ^{a,b}
Visc_Pap	8.23 ± 1.57 ^b	9.03 ± 0.52 ^d	0.433 ± 0.038 ^d	0.065 ± 0.006 ^b
Flav	5.72 ± 0.81 ^a	6.07 ± 0.30 ^{b,c}	0.194 ± 0.009 ^b	0.041 ± 0.004 ^a
Pap	6.37 ± 0.39 ^a	6.88 ± 0.64 ^{b,c}	0.289 ± 0.003 ^c	0.037 ± 0.003 ^a
Control	6.03 ± 0.59 ^a	4.65 ± 0.38 ^a	0.129 ± 0.009 ^a	0.049 ± 0.004 ^a
<i>P. dioica</i>				
Visc	14.30 ± 0.95 ^b	6.98 ± 0.67 ^b	0.427 ± 0.015 ^a	0.044 ± 0.001 ^a
Visc_Flav	18.27 ± 1.87 ^c	10.1 ± 0.68 ^c	0.567 ± 0.024 ^b	0.053 ± 0.005 ^{a,b}
Visc_Pap	17.02 ± 2.25 ^c	14.1 ± 1.80 ^d	0.639 ± 0.049 ^c	0.070 ± 0.006 ^b
Flav	10.73 ± 1.41 ^a	6.76 ± 0.62 ^b	0.435 ± 0.034 ^a	0.044 ± 0.004 ^a
Pap	10.43 ± 0.79 ^a	12.3 ± 0.54 ^d	0.627 ± 0.014 ^c	0.056 ± 0.004 ^a
Control	10.68 ± 0.21 ^a	5.22 ± 0.49 ^a	0.408 ± 0.011 ^a	0.046 ± 0.004 ^a
<i>U. rigida</i>				
Visc	9.74 ± 1.72 ^b	4.56 ± 0.43 ^c	0.241 ± 0.013 ^b	0.130 ± 0.015 ^b
Visc_Flav	17.35 ± 0.16 ^c	6.56 ± 0.64 ^d	0.360 ± 0.023 ^c	0.150 ± 0.006 ^b
Visc_Pap	16.56 ± 1.33 ^c	8.16 ± 0.31 ^e	0.390 ± 0.032 ^c	0.142 ± 0.018 ^b
Flav	1.97 ± 0.10 ^a	3.27 ± 0.32 ^b	0.125 ± 0.005 ^a	0.090 ± 0.011 ^a
Pap	1.71 ± 0.63 ^a	5.65 ± 0.24 ^d	0.211 ± 0.006 ^b	0.096 ± 0.007 ^a
Control	1.76 ± 0.05 ^a	2.00 ± 0.17 ^a	0.087 ± 0.003 ^a	0.095 ± 0.010 ^a
<i>F. vesiculosus</i>				
Visc	14.07 ± 0.33 ^c	1.80 ± 0.08 ^c	0.514 ± 0.044 ^b	0.161 ± 0.014 ^{b,c}
Visc_Flav	13.11 ± 0.61 ^c	2.94 ± 0.13 ^d	0.590 ± 0.024 ^c	0.175 ± 0.007 ^c
Visc_Pap	10.58 ± 1.29 ^a	3.67 ± 0.06 ^e	0.599 ± 0.026 ^c	0.163 ± 0.011 ^{b,c}
Flav	10.47 ± 1.16 ^a	1.44 ± 0.05 ^b	0.511 ± 0.015 ^b	0.144 ± 0.016 ^b
Pap	12.10 ± 0.22 ^a	1.65 ± 0.06 ^c	0.486 ± 0.006 ^b	0.128 ± 0.011 ^a
Control	11.92 ± 0.30 ^b	0.88 ± 0.08 ^a	0.371 ± 0.014 ^a	0.128 ± 0.007 ^a

Results obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap) and presented as mean ± SD. Different letters indicate significant differences between extracts for each seaweed at $p < 0.05$.

Bioactive peptides have gained interest in different fields, namely for functional foods, nutraceuticals, cosmetic or pharmaceutical applications. In the present work, proteins and peptides profiles were analyzed by size exclusion chromatography and are shown in Fig. 3. The peptide/protein profile shows one to two main peaks near or at 1200 Da. The profile clearly shows the protein hydrolysis during the process of extraction with the application of enzymes, demonstrated by the decrease of the main peaks and generation of lower intermediate MW species. Results demonstrate that a combination of carbohydrases and proteases proved to increase protein solubilization, protein hydrolysis to peptides and increased antioxidant activity in extracts subjected to the combination of enzymes. This may lead to technological advances, increases in bioavailability, digestibility and bioactive potential.

3.5. Antioxidant activity

Over the past years, dietary oligomers, polyphenols and peptides from seaweeds have been widely studied for their biological activities including antioxidant activity, anti-inflammation, antibacterial, anticancer prebiotic to immunomodulatory among others (Cheong et al., 2018; Cotas et al., 2020).

The antioxidant activity of the seaweed extracts was assessed by three different *in vitro* assays. Results have shown that antioxidant activity varied with the application of enzymes, as well as with the seaweed species. *F. vesiculosus* presented the highest antiradical scavenging activity, in all

antioxidant assays, being this correlated with its higher total phenolic compounds. As shown in Fig. 4, both sequential treatment with Viscozyme and Flavourzyme and Viscozyme and papain exhibited a higher radical scavenging effect than when the enzymes were used separately. The increase in the antioxidant activity seems to be correlated not only with the phenolic compounds but also with the release of peptides and oligomers within the hydrolysis of substrate by both proteases and carbohydrases. The increase in the antioxidant activity has been correlated with an increase in polyphenols (Wang et al., 2009), protein hydrolysates (Cian et al., 2015b) and oligosaccharides (Cheong et al., 2018) from different seaweeds.

Antioxidant extracts may be interesting for industrial applications, such as the development of functional foods. However, the antioxidant activity must be maintained after being ingested and submitted to the gastrointestinal (GI) tract under adverse conditions (Cunha and Pintado, 2022). Based on this, the two most promising seaweed extracts (*G. vermiculophylla* and *P. dioica*) were submitted to an *in vitro* GI simulation. In order to understand the effect of each step of the GI digestion in the extracts, the antioxidant activity/capacity was measured by the ABTS and ORAC. In both methods, a slight increase in the antioxidant activity after the steps of the GI digestion was observed, especially after small intestine simulation (Data not shown). This could be an interesting result, since these extracts may be incorporated into functional foods without compromising their antioxidant properties after GI digestion.

Table 6 – Carbohydrate profile of the extracts in the different seaweeds.

Seaweed	Uronic acid units (g/L)	Glucose units (g/L)	XMG units (g/L)	Rhamnose units (g/L)	Arabinose units (g/L)	Fucose units (g/L)
<i>G. vermiculophylla</i>						
Visc	0.252 ± 0.018 ^a	1.350 ± 0.021 ^c	1.385 ± 0.063 ^b	-	0.174 ± 0.018 ^a	-
Visc_Flav	0.209 ± 0.064 ^a	1.452 ± 0.027 ^c	1.512 ± 0.107 ^b	-	0.187 ± 0.007 ^a	-
Visc_Pap	0.267 ± 0.013 ^a	1.039 ± 0.241 ^b	1.068 ± 0.178 ^a	-	0.146 ± 0.021 ^a	-
Flav	0.620 ± 0.098 ^b	0.091 ± 0.007 ^a	1.029 ± 0.148 ^a	-	-	-
Pap	0.569 ± 0.079 ^b	0.089 ± 0.021 ^a	1.296 ± 0.043 ^{a,b}	-	-	-
Control	0.541 ± 0.107 ^b	0.111 ± 0.008 ^a	1.182 ± 0.082 ^{a,b}	-	-	-
<i>P. dioica</i>						
Visc	0.278 ± 0.093 ^a	0.222 ± 0.059 ^a	3.484 ± 0.356 ^b	-	0.385 ± 0.050 ^a	-
Visc_Flav	0.395 ± 0.056 ^a	0.117 ± 0.003 ^a	4.551 ± 0.527 ^c	-	0.348 ± 0.012 ^a	-
Visc_Pap	0.422 ± 0.021 ^a	0.139 ± 0.001 ^a	4.133 ± 0.644 ^c	-	0.356 ± 0.008 ^a	-
Flav	0.391 ± 0.032 ^a	-	2.762 ± 0.383 ^a	-	-	-
Pap	0.364 ± 0.061 ^a	-	2.701 ± 0.239 ^a	-	-	-
Control	0.459 ± 0.052 ^a	-	2.681 ± 0.079 ^a	-	-	-
<i>U. rigida</i>						
Visc	0.349 ± 0.172 ^a	0.815 ± 0.022 ^b	0.191 ± 0.032 ^a	1.410 ± 0.308 ^b	-	-
Visc_Flav	0.204 ± 0.004 ^a	2.542 ± 0.170 ^c	0.266 ± 0.008 ^a	1.916 ± 0.122 ^c	-	-
Visc_Pap	0.209 ± 0.018 ^a	2.346 ± 0.233 ^c	0.206 ± 0.010 ^a	1.943 ± 0.138 ^c	-	-
Flav	0.153 ± 0.006 ^a	0.258 ± 0.016 ^a	-	0.148 ± 0.031 ^a	-	-
Pap	0.147 ± 0.025 ^a	0.237 ± 0.068 ^a	-	0.180 ± 0.014 ^a	-	-
Control	0.115 ± 0.029 ^a	0.231 ± 0.013 ^a	-	0.155 ± 0.028 ^a	-	-
<i>F. vesiculosus</i>						
Visc	3.189 ± 0.033 ^b	0.081 ± 0.009 ^a	0.383 ± 0.042 ^a	-	-	0.485 ± 0.028 ^a
Visc_Flav	2.954 ± 0.136 ^b	0.086 ± 0.017 ^a	0.323 ± 0.062 ^a	-	-	0.493 ± 0.012 ^a
Visc_Pap	3.111 ± 0.274 ^{a,b}	0.081 ± 0.010 ^a	0.328 ± 0.048 ^a	-	-	0.488 ± 0.061 ^a
Flav	2.406 ± 0.194 ^a	0.124 ± 0.075 ^a	0.322 ± 0.011 ^a	-	-	0.333 ± 0.066 ^a
Pap	2.659 ± 0.035 ^a	0.101 ± 0.004 ^a	0.342 ± 0.011 ^a	-	-	0.457 ± 0.015 ^a
Control	2.726 ± 0.121 ^a	0.083 ± 0.023 ^a	0.294 ± 0.023 ^a	-	-	0.402 ± 0.055 ^a

Results obtained by enzyme-assisted extraction with Viscozyme® L (Visc), Flavourzyme® (Flav) and papain (Pap) and presented as mean ± SD. Different letters indicate significant differences between extracts for each seaweed at $p < 0.05$.

3.6. Prebiotic potential

Recent studies have demonstrated the prebiotic potential of marine resources, namely, seaweeds. These effects have been attributed to its richness in complex polysaccharides that can be resistant to digestion by enzymes present in the human gastrointestinal tract, and selectively stimulate the growth of beneficial gut bacteria (Charoensiddhi et al., 2017a; Cian et al., 2015a). Likewise, ingested polyphenols, proteins/peptides can also reach the large intestine where they can be converted into beneficial bioactive metabolites by microbiota (Cian et al., 2015a; Echave et al., 2021). Nonetheless, in the literature, no information is available regarding the prebiotic potential of extracts obtained by the combination of carbohydrases and proteases targeted in this study.

For the evaluation of the prebiotic effect the two red seaweeds, *G. vermiculophylla* and *P. dioica* were chosen in the treatment condition that induced the highest carbohydrate and proteins/peptides release that was the pre-treatment with Viscozyme followed by treatment with Flavourzyme.

With regard to media with a carbon source (Fig. 5), in Bb12, significantly higher values ($p < 0.05$) of growth were observed for media enriched with 2% of digested and non-digested *G. vermiculophylla* extract and 2% of non-digested and 1% of digested *P. dioica* extract in comparison with negative control. In Bo, growth was significantly increased in media supplemented with 1% of non-digested *G. vermiculophylla* extract and 2% of digested *P. dioica* extract. Whereas in the LAS strain, a significant inhibition in cell growth was observed for both extracts at the different concentrations tested. The opposite was observed in the *L. casei* strain where

a strong prebiotic effect was observed in both extracts for all concentrations, apart from 2% of digested *P. dioica*.

Results of incorporation of extracts in MRS medium without glucose supplementation are presented in Fig. 6. In Bb12, the incorporation of extracts showed a positive effect in the conditions of 1% non-digested and digested *G. vermiculophylla* extract and 1% and 2% non-digested *P. dioica*. Similar results were observed in the Bo strain after incubation with non-digested extracts of *G. vermiculophylla* and *P. dioica* and 2% of digested *P. dioica*. Greater results were observed in *L. casei*, where the growth was significantly enhanced in all media incorporated with extract in comparison with MRS without sugar source (negative control). By opposite, LAS growth was not positively affected by the presence of any of the tested extracts; in fact, the growth of this bacterium seems to be decreased by the presence of the extracts. This confirms how the impact of prebiotic ingredients is strain-dependent.

Concerning the incorporation of FOS in MRS without a carbon source, a significant increase in bacterial growth was observed in all strains, except for the LAS strain. Overall, a similar bifidogenic effect with FOS was observed in *G. vermiculophylla* and *P. dioica* extracts. This effect was maintained in 1% of *G. vermiculophylla* extract after digestion. A greater prebiotic effect was obtained in *L. casei* strain in *G. vermiculophylla* extracts in both digested and non-digested samples, in comparison with the positive control of FOS. In *P. dioica*, the prebiotic effect was like those obtained in FOS. This effect is probably due to polysaccharides and oligosaccharides derived from hydrocolloids present in each seaweed that can act as sources of soluble fibers exerting a prebiotic effect.

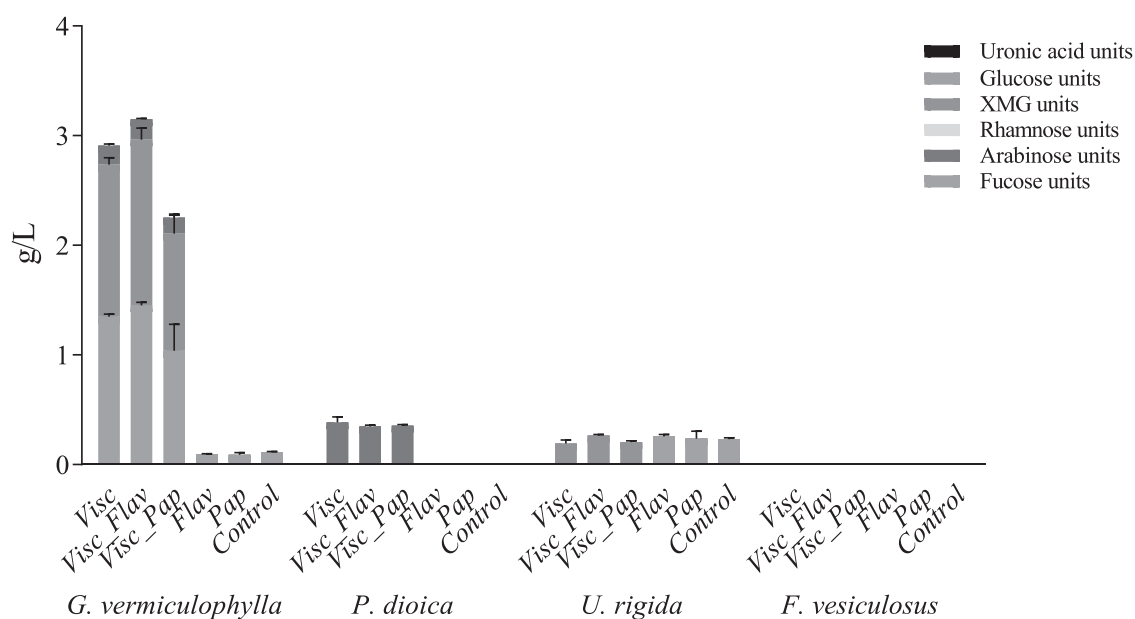


Fig. 2 – Oligosaccharide/low molecular weight polysaccharide profile of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction.

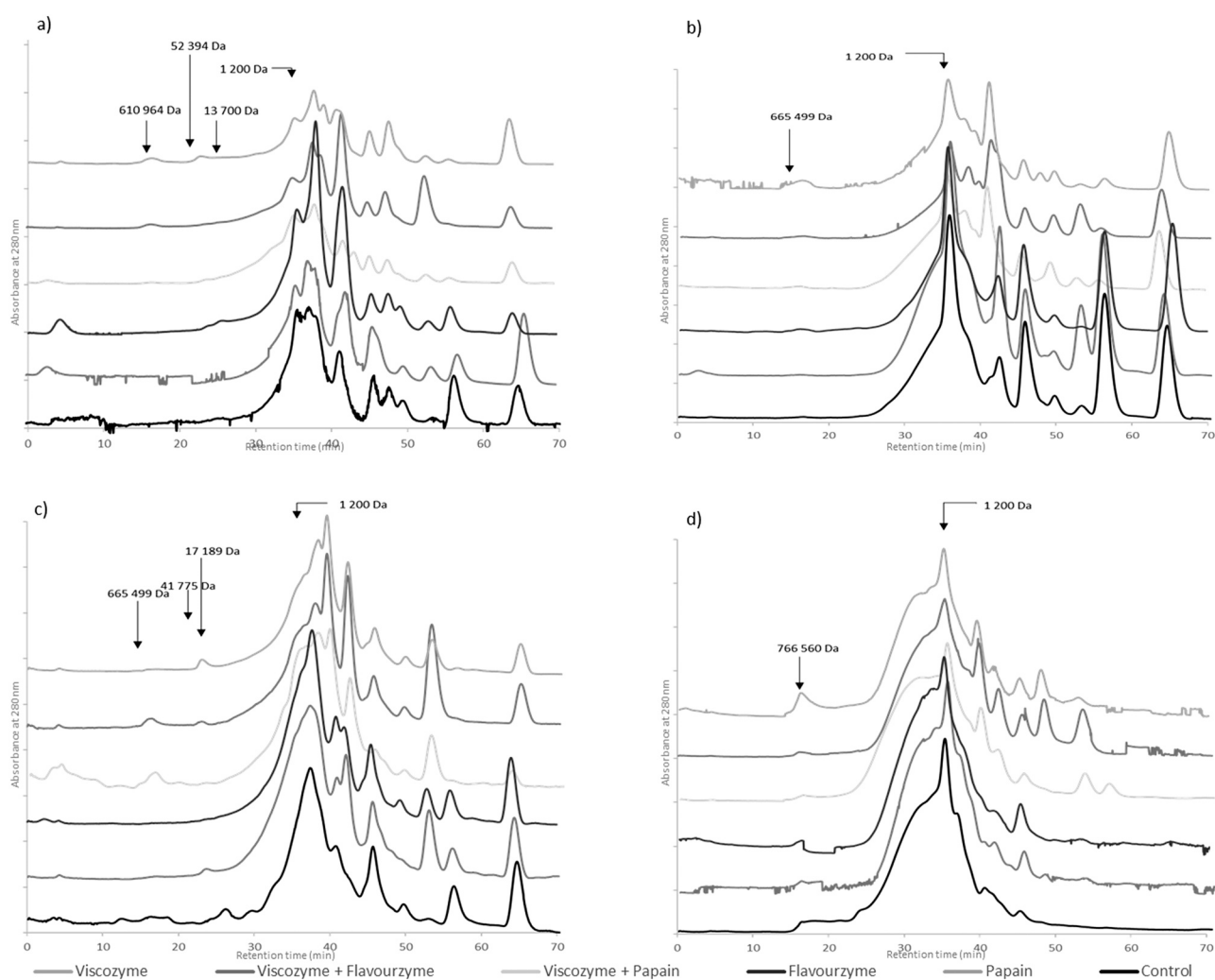


Fig. 3 – Peptide profile analysed by FPLC of *G. vermiculophylla* (a), *P. dioica* (b), *U. rigida* (c) and *F. vesiculosus* (d) extracts obtained by enzyme-assisted extraction.

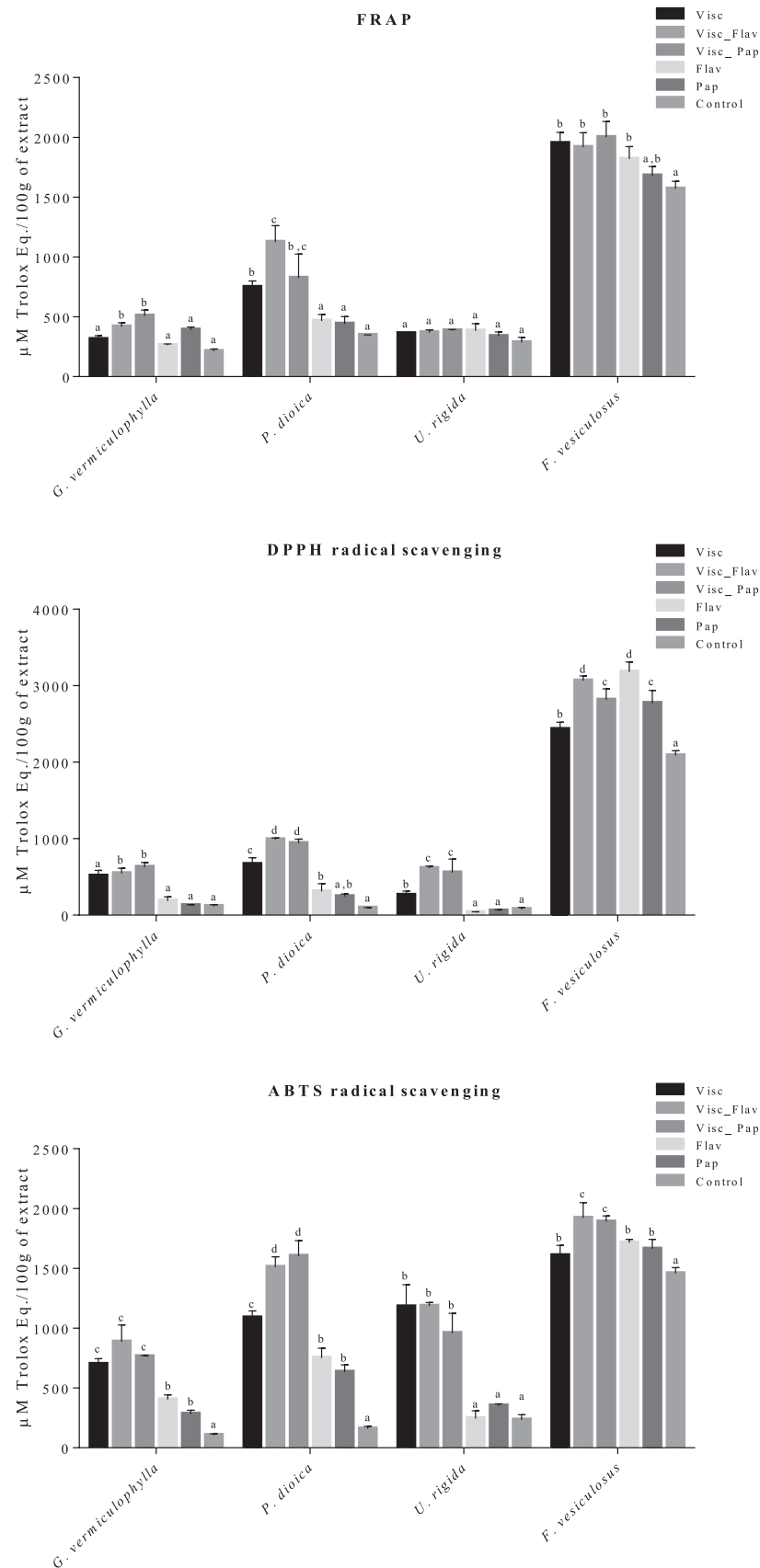


Fig. 4 – Antioxidant activity assessed by FRAP, DPPH and ABTS radical scavenging of *G. vermiculophylla*, *P. dioica*, *U. rigida* and *F. vesiculosus* extracts obtained by enzyme-assisted extraction. Different letters indicate significant differences between extracts for each seaweed at $p < 0.05$.

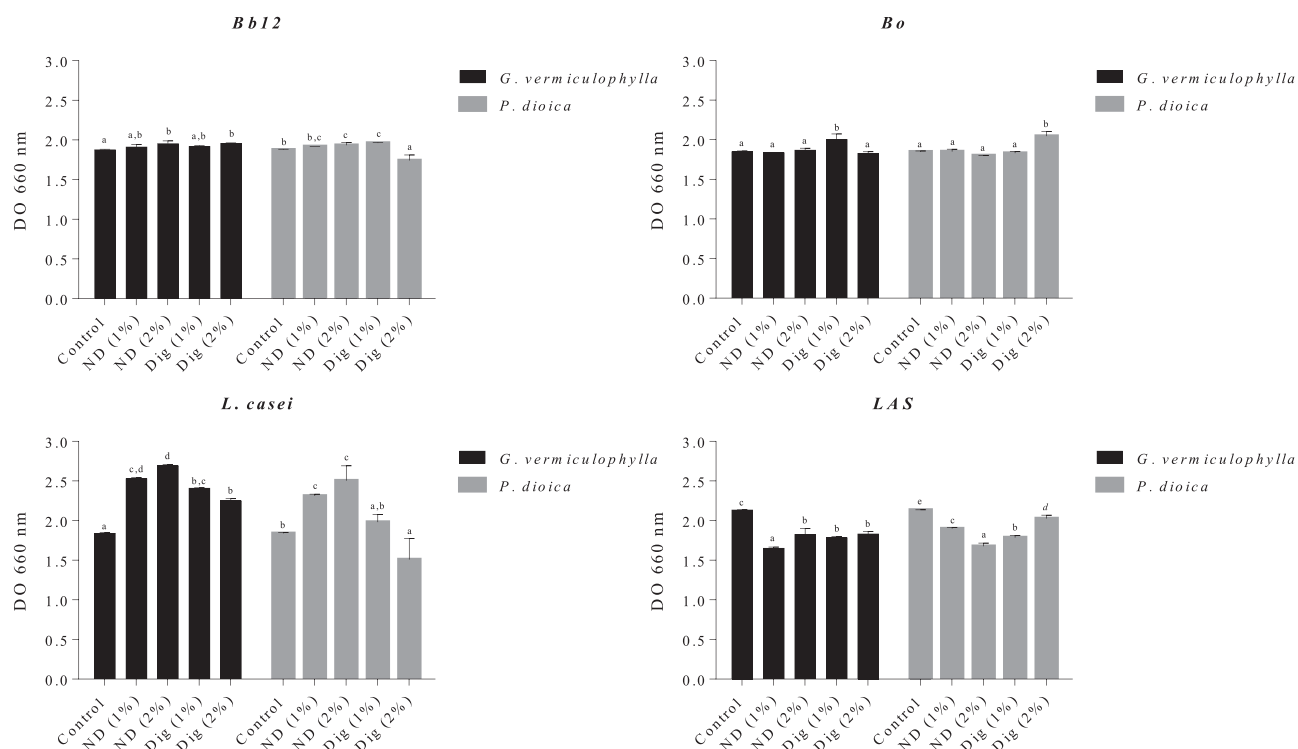


Fig. 5 – Maximum growth obtained for *Bifidobacterium animalis* spp. *lactis* (Bb12), *Bifidobacterium animalis* (Bo), *Lactobacillus casei* (*L. casei*) L26 and *Lactobacillus acidophilus* Ki (LAS) strains in the presence of glucose (Glc) (growth control), *G. vermiculophylla* digested (Dig) and non-digested (ND) (1% and 2%), and *P. dioica* digested and non-digested (1% and 2%). Tests with the samples were performed in MRS with 2% of glucose. The analyses were performed in triplicate and data are expressed as mean \pm SD of the replicates.

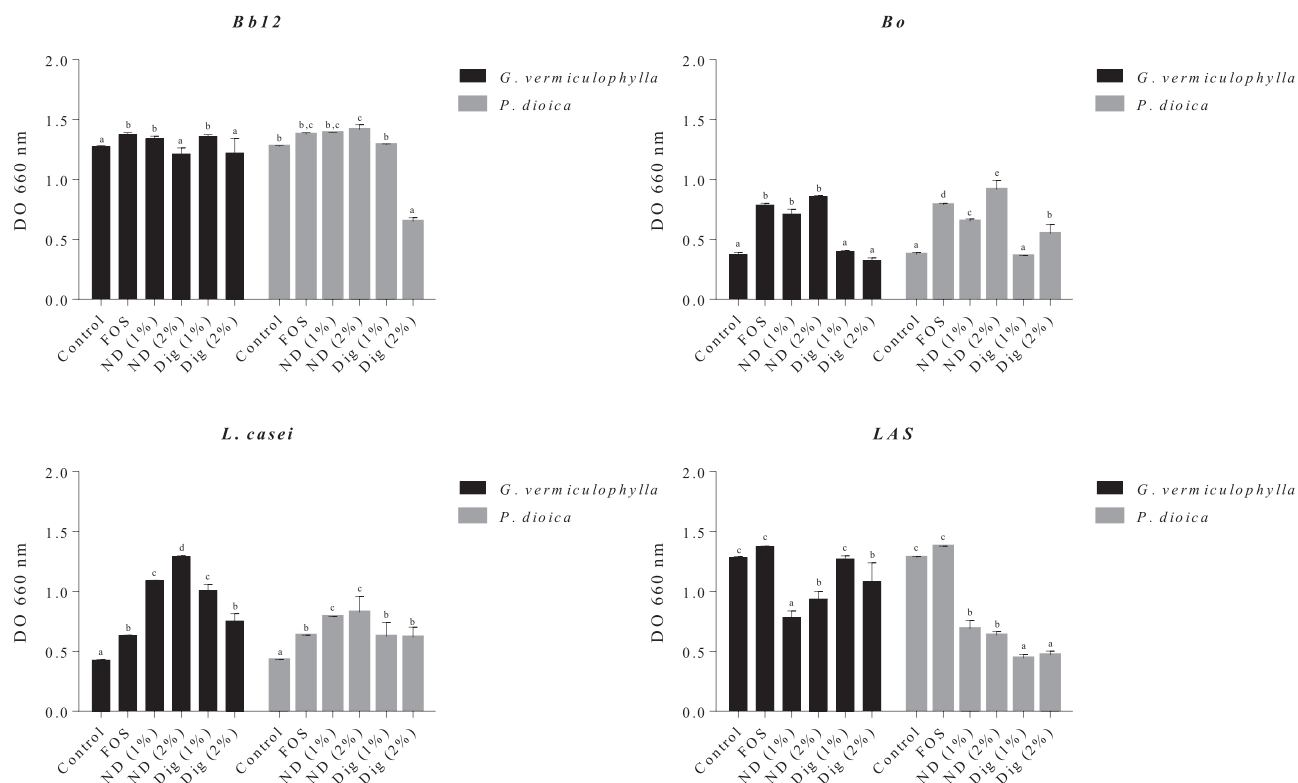


Fig. 6 – Maximum growth obtained for *Bifidobacterium animalis* spp. *lactis* (Bb12), *Bifidobacterium animalis* (Bo), *Lactobacillus casei* (*L. casei*) L26 and *Lactobacillus acidophilus* Ki (LAS) strains in MRS without a sugar source (growth control), FOS (positive control), *Gracilaria* digested (Dig) and non-digested (ND) (1% and 2%), and *Porphyra* digested and non-digested (1% and 2%). The tests with the samples were performed in MRS without a sugar source.

Prebiotic effects were reported in *B. animalis* and *L. acidophilus* La-5 when incubated with extracts obtained by ultrasound-assisted extraction with Viscozyme in *Sargassum muticum*, *Osmundea pinnatifida*, and *Codium tomentosum* (Rodrigues et al., 2015). Polysaccharide extracts of *Gracilaria* sp. extracted with Cellulase R-10 and Macerozyme R-10 also showed bifidogenic effects by increasing colony formation of *B. longum* BCRC 11847 (Shao-Chi et al., 2012). In both studies, the investigation of the effect of GI digestion was not performed.

In vitro studies using an anaerobic human fecal fermentation model for gut fermentation processes also reported that laminarin could stimulate the production of short-chain fatty acids (SCFA), metabolites produced by a healthy microbiota (Deville et al., 2007). Low molecular weight polysaccharides from the red seaweed *Gelidium sesquipedale* induced a significant increase in populations of *Bifidobacterium*, as well as an increase in acetate and propionate (Ramnani et al., 2012). A similar result using an extract from the brown seaweed *Osmundea pinnatifida* was reported by Rodrigues et al. (2016). Charoensiddhi et al., 2016, 2017b also demonstrated that extracts from the brown seaweed *Ecklonia radiata* stimulated the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacilli* and SCFA production.

4. Conclusions

Seaweeds are a valuable source of bioactive compounds, mainly poly-, oligo- and monosaccharides, proteins and peptides and phenolic compounds. The potential of these compounds for several industries, particularly in the food industry, has been increasingly recognized.

A significant improvement in the extraction yield of carbohydrates, proteins, and phenolic compounds was here reported with a sequential treatment with the use of carbohydrase followed by proteases. Particularly, the combination of Viscozyme and papain proved to be more efficient for the extraction of protein, whereas Viscozyme and Flavourzyme in the extraction of carbohydrates. In addition, an increase in polyphenols, protein hydrolysates and oligosaccharides in the different enzyme-assisted extracts was observed. This increased the antioxidant activity of extracts, being maintained even after gastrointestinal digestion simulation. Extracts from red seaweeds also demonstrated prebiotic potential showing an increase in cell growth of *Lactobacillus* and *Bifidobacterium* strains.

Overall, this study demonstrates that these edible seaweeds are natural sources of valuable compounds with important biological interest. The combination of cellulolytic and proteolytic enzymes had a synergistic effect on the extraction of the different classes of compounds and was revealed to be a promising tool to obtain extracts with attractive biological properties. These results envisage that advances in enzymatic processes are a key trend for efficient extraction of added-value bioactive compounds from seaweeds. However, future studies should be performed regarding the development of functional food and nutraceutical.

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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