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# *In vitro* digestibility and Caco-2 cell bioavailability of sea lettuce (*Ulva fenestrata*) proteins extracted using pH-shift processing

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ABSTRACT

Seaweed is a promising sustainable source of vegan protein as its farming does not require arable land, pesticides/insecticides, nor freshwater supply. However, to be explored as a novel protein source the content and nutritional quality of protein in seaweed need to be improved. We assessed the influence of pH-shift processing on protein degree of hydrolysis (%DH), protein/peptide size distribution, accessibility, and cell bioavailability of *Ulva fenestrata* proteins after *in vitro* gastrointestinal digestion. pH-shift processing of *Ulva*, which concentrated its proteins 3.5-times, significantly improved the %DH from 27.7±2.6% to 35.7±2.1% and the amino acid accessibility from 56.9±4.1% to 72.7±0.6%. Due to the higher amino acid accessibility, the amount of most amino acids transported across the cell monolayers was higher in the protein extracts. Regarding bioavailability, both *Ulva* and protein extracts were as bioavailable as casein. The protein/peptide molecular size distribution after digestion did not disclose a clear association with bioavailability.

## 1. Introduction

Consumption of alternative protein sources is forecasted to grow by an annual rate of 9% until 2054 (Probst, Frideres, Pedersen, & Amato, 2015); seaweeds can play an important role in the ongoing protein shift as - unlike terrestrial protein crops - its cultivation does not compete for arable soil or freshwater supply. In fact, between 2024 and 2054 the alternative protein market share of algae, i.e. macro- and microalgae, is expected to increase by 16% (Probst et al., 2015). To meet the economieś fast-growing demand for seaweed biomass (macroalgae), seaweed aquaculture at both, open-water and on-shore tank cultivation systems, is a more sustainable and profitable alternative when compared to the harvest of wild seaweed populations. We recently reported on successful tank cultivation of *Ulva fenestrata* Postels & Ruprecht (Toth et al., 2020).

Although seaweed can contain all essential amino acids at various concentrations (Abdollahi et al., 2019), most seaweed species, apart from a few red seaweed species like *Porphyra tenera* (Holdt & Kraan, 2011), have a relatively low protein content (9–22% DW) compared to terrestrial vegetable-protein sources such as soybean (48–52% DW) and lupine (39–55% DW) (Bähr, Fechner, Hasenkopf, Mittermaier, & Jahreis, 2014; Holdt & Kraan, 2011). Additionally, its digestibility can be negatively affected by the presence of structures containing e.g. polysaccharides and phenolics (Fleurence, Morançais, & Dumay, 2017; Tibbetts, Milley, & Lall, 2016). Thus, to fully explore seaweed as a protein source, efforts need to be spent on developing extraction methods to concentrate its protein and to remove potential anti-nutrients.

Among the main protein extraction methods reported for seaweed, the pH-shift method with protein solubilization at pH 12, with and without an extra incubation at pH 8.5, has proven to achieve the highest protein yields and protein purities for *Saccharina*, *Porphyra* and *Ulva*. Moreover, it is a relatively easy technology to scale-up when compared to traditional methods e.g. sonication in water followed by ammonium sulphate-induced protein precipitation (Harrysson et al., 2018; 2019). The pH-shift process is based on the principle that depending on the pH of a protein-water mixture, a protein will have a variable net charge and thus a different solubility. With solubilization done at alkaline pHs and precipitation at acidic pHs, this method has been used to extract protein from brown (Abdollahi et al., 2019; Harrysson et al., 2018; Veide Vilg & Undeland, 2017), red (Harrysson et al., 2018), and green seaweeds

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(Angell, Paul, & de Nys, 2017; Harrysson et al., 2018; 2019). To be best of our knowledge, it is not known how such extractions affect protein digestibility and bioavailability. Also, the co-extraction of seaweed antinutrients alongside proteins using different extraction methods remains little explored. So, we hypothesized that structural disintegration and partial removal of antinutritional factors during protein extraction would improve digestibility and bioavailability.

Protein digestibility corresponds to the release of free amino acids and/or peptides from food during passage through the gastrointestinal tract (Guerra et al., 2012). As far as we know, *in vivo* studies of protein digestibility have only been performed on crude seaweeds. A sheep model revealed that a diet supplemented with *Saccharina latissima* decreased nitrogen absorption when compared to diets containing *Porphyra* sp. or soybean (Özkan Gülzari, Lind, Aasen, & Steinshamn, 2019). However, a study on rats pointed towards no differences between seaweed supplemented and regular rodent diets (Taboada, Millán, & Miguez, 2013). *In vitro* studies on protein digestibility, which indeed reduce costs and avoid ethical constraints (Guerra et al., 2012), have so far addressed either crude seaweed (Tibbetts et al., 2016) or seaweed protein extracts (Gajaria et al., 2017; Kazir et al., 2019; Wong & Cheung, 2001). Yet, to the best of our knowledge, extracts and crude seaweed have not been compared yet.

Protein bioavailability is defined as the fraction of digested protein that is transported from the intestinal lumen to the bloodstream (Xu, Hong, Wu, & Yan, 2019). The human intestinal Caco-2 cell line has been used over the years to study *in vitro* bioavailability of food components (Pongrac et al., 2016). Caco-2 cells are originally colonic enterocytes, but cultured post-confluence they differentiate into duodenal enterocytes. This differentiation happens because they grow in monolayers that share many morphological and functional traits with the human intestinal mucosa, such as cell polarization, microvillous structure, nutrient transport mechanisms, and expression of brush-border enzymes (Kamiloglu, Capanoglu, Grootaert, & Van Camp, 2015; Xu, Hong, Wu, & Yan, 2019). Previous studies have used Caco-2 cells to evaluate amino acid bioavailability from chickpea and lupin (Rubio & Clemente, 2009), soy (Mcgraw et al., 2014), whey (Goulart et al., 2014), and collagen (Feng & Betti, 2017); but so far, no studies have reported on amino acids from seaweed.

The main goal of this study was to assess the influence of pH-shift processing on the *in vitro* digestibility (degree of hydrolysis [%DH] and protein/peptide molecular size distribution), accessibility, and Caco-2 cell bioavailability of *U. fenestrata* proteins. Other objectives included examining how these parameters were effected by (i) freeze-drying since vegetarian protein extracts are often commercialized in powder form and (ii), seaweed species focusing on a comparison between *U. fenestrata* and the more commonly cultivated *S. latissima* that has a lower protein content and a higher level of phenolics (Holdt & Kraan, 2011).

## 2. Materials and methods

### 2.1. Seaweed cultivation

Biomass of the northern hemisphere Sea Lettuce, *Ulva fenestrata* - previously known as *Ulva lactuca* L. - was taken from a long-term indoor tank culture at the Tjärnö Marine Laboratory (58°52′36.4″N 11°6′42.84″E). The seaweed was grown in a green-house within 90 L cultivation tanks and was exposed to a permanent flow-through (flow = 8–10 L $h^{-1}$) of filtrated (0–5 μm + UV-light application) deep-sea (40 m) seawater. Permanent aeration, as well as light at an irradiance of 120 μmol $m^{-2}$ $s^{-1}$ (light source: INDY66 LED 60 W 4000 K 6000 lm) and at a 16:8h (L:D) photo regime, was applied to the cultures. Additionally, once per week, the seaweed obtained a nutrient media solution (Provasoli Enriched Seawater). The flow-through was interrupted for 2 h after the media was applied, to facilitate nutrient assimilation of the biomass. The salinity and temperature fluctuated depending on the prevailing weather and seasonal conditions. The harvest of biomass used within this study took place in November 2019. The brown seaweed *S. latissima* was provided by the same marine laboratory (Tjärnö, Sweden), where it was tank-cultivated and harvested in June 2018.

### 2.2. Taxonomic identification

The used *Ulva* strain was taxonomically identified by DNA barcoding of the tufa marker gene. The identification followed the procedure explained by Toth et al. (2020). Sequences of the strain (MN240309-MN240311) are publicly available at the online database GenBank.

### 2.3. Seaweed preparation and extraction of proteins using the pH-shift process

Fresh and crude *U. fenestrata and S. latissima* were kept on ice after harvest and minced within a day using a meat grinder (Model C-E22N, la Minerva) with a 4.5 mm hole plate and stored at − 80 °C in Ziplock bags until further use. Upon use, the biomass was thawed under running cold water.

The production of protein extracts from *U. fenestrata* using the pH-shift method followed the protocol reported by Harrysson et al. (2019) with some modifications. Due to the relatively large amount of protein extract required for all analyses and the fact that we only had access to lab-scale equipment, the pH-shift process had to be divided into two runs. The produced protein extracts were however pooled into one large batch which was then sub-divided for the triplicate *in vitro* digestions. This was to separate the variance in the digestion and cell trial steps from the variance caused by the pH-shift process step as such. Our experience with pH-shift processing of seaweed has earlier shown that a third replica does not change the variability in the results and that the composition of protein extracts produced in separate runs is very even, with relative standard deviations (RSD%) in total protein and total fatty acids contents of<5 and 8%, respectively (Abdollahi et al., 2019; Harrysson et al., 2018). Very little is however known about the experimental variation when digesting seaweed and subjecting such digests to transport through Caco-2 cells. In each pH-shift run, a representative amount of minced *U. fenestrata* (600 g) was mixed with distilled water in a 1:6 (w/v) ratio. Homogenization was done using a Silverson LM5 for 4 min at 8000 rpm, followed by an osmoshock incubation step for 1 h at 8 °C with stirring. Thereafter, the pH of the homogenate was adjusted to 8.5 (PHM 210, Meterlab, Hach) with NaOH (2 M) and left to incubate for 1 h while stirring. To solubilize the maximum amount of proteins, the pH was then adjusted to 12.0 using NaOH (2 M) and left to incubate for 20 min while stirring, followed by centrifugation at 8000 × *g* for 20 min at 8 °C (Sorvall LYNX 6000, Thermo Scientific). The resulting supernatant containing the solubilized proteins was decanted through a sieve (~0.5 mm) and its pH was adjusted to 3.0 with HCl (1 M). Afterwards, the supernatant was frozen overnight at − 20 °C and was thawed in cold water before centrifugation at 8000 × *g* for 20 min at 8 °C. The resulting pellets (i.e. the protein extract) from both batches were mixed and stored at − 80 °C. A part of it was thereafter subjected to freeze-drying. During the pH-shift process, samples were withdrawn from the homogenate and supernatants 1 and 2 (S1 and S2) for protein analysis. The whole process was performed on ice unless stated otherwise.

### 2.4. Total phenolic content

The extraction of total phenolic compounds was performed according to Barnes et al. (2009) with some modifications. Briefly, all samples were freeze-dried and milled with a mortar and pestle to a fine powder. Thereafter, crude *S. latissima* (n = 3), crude *U. fenestrata* (n = 2) and, and pH-shift extract from *U. fenestrata* (n = 2) were weighed and extracted with 5 mL of acidified methanol [MeOH:$H_2O$ (70:30) + 1% trifluoracetic acid]. The samples were sonicated for 10 min with a shaking step after 5 min sonication. Then, the samples were incubated for 30 min in a water bath at 60 °C, followed by a centrifugation step (5000x*g* for 5 min). The resultant supernatant was collected, and the pellet was resuspended in acidified methanol; but without the previously described incubation step at 60 °C. Both supernatants were pooled and stored at −20 °C. The total phenolics content of the extracts was evaluated by the Folin–Ciocalteu method with slight modifications as reported by Trigo et al. (2020). The samples were analysed in triplicate and the results were expressed as mg phloroglucinol equivalents/g sample DW.

### 2.5. In-vitro gastrointestinal digestion

Based on preliminary trials, we selected a non-cytotoxic amount of protein to be digested and all digestions were done on an equal protein basis, unless stated otherwise. Crude protein content was determined based on total nitrogen (Section 2.9.2). These nitrogen estimations generally correlated well with the total amino acid analysis (Section 2.9.3); a small deviation between both analyses was detected for crude *U. fenestrata* though.

Before starting the digestion, the activity of porcine pepsin (P7012, Sigma) and porcine pancreatin (P7545, Sigma) as well as the total content of bile acids in the porcine bile extract (B8631, Sigma) was measured and compared to the values stated by the manufacturer. The simulated gastrointestinal digestions followed the protocol recommended by Latunde-Dada, Yang, and Vera Aviles (2016) with some modifications. Minced wet *U. fenestrata* and *S. latissima*, wet and freeze-dried pH-shift extract from *U. fenestrata*, and casein corresponding to 67 or 33 mg crude protein were weighed into 50 mL Falcon tubes and 4 mL of isotonic saline solution tempered to 37 °C (140 mM NaCl and 5 mM KCl) was added to the tubes. The lower amount of protein to be digested (i.e. 33 mg) allowed to test if the digestive enzymes were saturated with substrate and if the apical-to-basolateral transport was within the linear regime of dose–response. A digestion blank without sample was also included to measure the contribution of the digestive compounds (i.e. digestive enzymes) in the different measurements. To mimic, as close as possible, the mouth mastication, a polytron (Ultra-turrax T18 basic, IKA) was used for 10 s at a speed of 18 000 rpm. To recover as much sample as possible from the polytron, the equipment was run in a separate 2 mL portion of isotonic saline solution. The resulting solution was pooled with the initial sample and the pH was set to 7.0 with HCl (1 M) or $NaHCO_3$ (1 M). After adjusting the volume to 6.5 mL with isotonic saline solution, the tubes were pre-tempered for 15 min at 37 °C. At this step, samples (n = 1) were taken to measure the %DH and protein/peptide size distribution before digestion. Next, 0.5 mL of α-amylase solution (1050 U/mL in isotonic saline solution) was added, followed by an incubation step for 2 min at 37 °C with shaking at 160 rpm (Max Q 4000, Barnstead/Lab-line). For the gastric step, the pH was immediately lowered to ≤ 3 using HCl (1 M) in all samples to stop α-amylase activity. Then, the pH was fine-tuned to pH 2.0. Thereafter, sample volume was set to 9.5 mL with isotonic saline solution, and 0.5 mL of pepsin stock solution (40 000 U/mL 0.1 M HCl) was added. The tubes were incubated at 37 °C for 1 h. For the intestinal step, the pH was immediately raised to ≥ 5.5 using $NaHCO_3$ (1 M) in all samples to stop pepsin activity, then adjusted to 7.0. A volume of 0.5 mL of pancreatin stock solution (18.2 U/mL isotonic saline solution) and 2.0 mL of bile stock solution (10.625 mg/mL isotonic saline solution) were added and the total volume was brought to 15 mL with isotonic saline solution. The tubes were incubated as indicated above for 2 h. Aliquots for the bioavailability study were withdrawn and filtrated with a syringe filter (0.45 μm, Minisart RC, Sartorius) to recover the accessible fraction and to facilitate the recovery of cell lysates as larger fragments of the retentate could partially cover the cell monolayers. Thereafter, enzymes were heat-inactivated at 95 °C for 10 min in a heat block. After cooling to 37 °C, the aliquots were then used for Caco-2 cell bioavailability studies (Section 2.7) with the remaining volume of the digests being stored at − 80 °C. Each sample was digested in three separate digestions (n = 3).

### 2.6. Protein digestibility - determination of degree of hydrolysis

To measure the protein degree of hydrolysis before and after *in vitro* digestion, primary amines were detected according to Cavonius, Albers, and Undeland (2016). Aliquots of the digests (0.5 mL) were first centrifuged at 2000 × *g* for 5 min and then added to 4 mL of 1% SDS. Homogenization (Ultra-turrax T18 basic, IKA) was done for 10 s at a speed of 10 000 rpm. Then, to inactivate enzymes, all samples were heated at 75 °C for 15 min in a water bath. To each well of a 48-well plate (Costar, Corning), the following solutions were added in this order: 25 μL of sample, 200 μL of sodium phosphate buffer (0.2 M, pH 8.2), and 200 μL of freshly prepared TNBS solution (0.1% (w/v) in sodium phosphate buffer). The mixture was allowed to react in a heating cabinet (Max Q 4000, Barnstead/Lab-line) at 50 °C for 1 h with shaking (150 rpm) and the reaction was stopped by adding 400 μL of 0.1 M HCl. After cooling down the plate at room temperature for 30 min, the absorbance was measured at 420 nm in a Tecan Safire 2 plate reader with Magellan software version 5.03. Samples were analyzed in triplicate and a standard curve was made with DL-leucine. The degree of hydrolysis (%DH) was expressed according to Eq. (1):

$$\%DH = \frac{h(sample) - h(digestion\ blank)}{h_{tot}(sample)} \times 100 \quad (1)$$

where h is the sample's amount of primary amines (mmol leucine equivalents/g protein), and $h_{tot}$ is the maximum amount of primary amines in each sample (mmol/g protein) calculated through the amino acid profile.

### 2.7. Caco-2 cell culture and transport studies

Human intestinal Caco-2 cells (HTB-37; American Type Culture Collection) were purchased in passage 19 and grown in MEM (Lonza) supplemented with 10% fetal bovine serum (Gibco) and 0.2% Normocin (Invivogen) under 5% $CO_2$ humidified air at 37 °C (Heraeus HERAcell 150 Incubator, Thermo Electron Corporation). The cells were passaged at about 80% confluence and the medium was changed every second day, except for weekends. In passage 64–66, the cells were seeded on PET-transwell inserts ($1 \times 10^5$ cells/insert; pore size 0.4 μm, area 1.12 $cm^2$) in 12-well plates (Corning Life Sciences). After seeding, the cells were cultured for 14–15 days before the experiments.

On day 14 or 15, the medium was aspirated in both compartments and replaced by MEM supplemented with 1% PEST (Gibco). Then the cells were left in the incubator to acclimatize for 24 h. Thirty minutes before adding the digests, the medium was aspirated in both compartments and replaced by pre-heated HBSS at 37 °C. When adding the

digests, 0.250 mL of the apical HBSS was replaced by the same volume of digest. The cells were incubated for 2 h, after which the apical and basal HBSS were collected and stored at −80 °C. Then the cells were washed in phosphate-buffered saline (PAA Laboratories) and lysed in 0.100 mL of cold RIPA buffer (Sigma-Aldrich). The cell lysates were stored at −80 °C. Transport studies were replicated three times in separate experiments. In each experiment, a new batch of the digested sample was analyzed in triplicates (n = 9 in total). A cell control (i.e.blank without sample and digestive enzymes) was also included in the study layout.

### 2.8. Permeability of Caco-2 cell monolayers

The epithelial permeability was investigated by determining the content of zonulin – a tight function protein and a regulator of intestinal permeability - with a commercial ELISA kit (ElabScience). Zonulin standards and cell lysates (0.100 mL) were added to a microplate pre-coated with an antibody specific for zonulin and incubated for 90 min at 37 °C. Next, a biotinylated detection antibody specific for zonulin and an avidin-horseradish peroxidase conjugate was added to each well along with a substrate, and the cleavage was detected at 450 nm in a microplate reader. The zonulin content of the cells was expressed as a percentage of cell control (100%) and the results are provided in Supplementary Material: Fig. S1. Overall, no statistical differences were detected in all samples.

### 2.9. Total protein, total nitrogen and amino acid analysis

#### 2.9.1. Total protein

The Lowry method later modified by Markwell et al. (1978) was used to determine the total protein content of homogenate and supernatants of the pH-shift process. The total protein yield of the pH-shift process was then calculated according to Eq. (2).

$$Total\ yield\ (\%) = \frac{(Protein\ content\ of\ Supernatant\ 1 - Protein\ content\ of\ Supernatant\ 2)}{Protein\ content\ of\ Homogenate} \times 100 \quad (2)$$

#### 2.9.2. Total nitrogen

Total nitrogen of seaweed biomasses and protein extracts was analyzed using a Leco Protein Analyser (TruMac N, Leco Corporation). The traditional nitrogen-to-protein conversion factor of 5.0 was applied to calculate the protein content of both seaweeds, whereas the factor 6.25 was used for the pH-shift protein extract. The higher conversion factor for seaweed extracts is attributed to the removal of non-protein-bound nitrogen during the pH-shift processing.

#### 2.9.3. Total amino acids

Total amino acids of crude seaweeds, protein extracts, digests, and basal side media were determined according to the method reported by Abdollahi et al. (2019) with some modifications. To approximately 25–50 mg of freeze-dried sample, 10 mL of 6 M HCl was added; whereas for 1 mL of aqueous sample (digests and basal media), 8 mL of 6 M HCl and 1 mL of 12 M HCl were added. After replacing the air inside the tubes with nitrogen, samples were hydrolyzed (with caps on) at 110 °C for 24 h using a heat block. Hydrolyzed dried samples were diluted with 0.2 M acetic acid and filtered (0.22 μm; Fisher Scientific), while hydrolyzed aqueous samples were filtered (0.22 μm), flushed with nitrogen until dryness and subsequently resuspended in 0.2 M acetic acid. Two microliters of all samples were run in an LC/MS (Agilent 1100 HPLC) with a Phenomenex column (C18 (2) 250 μm × 4.6 μm × 3 μm), coupled to an Agilent 6120 quadrupole in the SIM positive mode (Agilent Technologies). The separation was conducted at 0.7 mL/min for 40 min using different ratios of mobile phase A (3% methanol, 0.2% formic acid, and 0.01% acetic acid) and mobile phase B (50% methanol, 0.2% formic acid and 0.01% acetic acid). For the calibration curve, a mix consisting of 17 amino acids (Thermo Scientific) was used. Due to the use of acidic hydrolysis, (i) tryptophan and cysteine could not be recovered and (ii) glutamine and asparagine were co-determined with glutamic and aspartic acid, respectively. Collected data were analyzed using the MassHunter Quantitive Analysis software (version B.09.00, Agilent Technologies).

#### 2.9.4. Calculation of amino acid accessibility and bioavailability

To calculate the amino acid accessibility and cell bioavailability Eqn. (3) and (4) were used, respectively.

$$Accessibility\ (\%) = \frac{Total\ amino\ acids\ of\ filtered\ digest}{Total\ amino\ acids\ before\ digestion + Total\ amino\ acids\ from\ digestive\ compounds} \times 100 \quad (3)$$

$$Bioavailability\ (\%) = \frac{Amount\ of\ amino\ acids\ in\ basolateral\ side\ after\ cell\ incubation}{Amount\ of\ amino\ acids\ in\ apical\ side\ right\ before\ cell\ incubation} \times 100 \quad (4)$$

### 2.10. Protein/peptide size relative distribution

The protein/peptide size relative distribution was evaluated with high-performance size-exclusion chromatography according to the method developed by Abdollahi et al. (2019) with some modifications to improve peak resolution. The instrument was constituted by a high performance size-exclusion chromatograph (HP-*SEC*) (Dionex HPLC, Dionex GmbH) equipped with two serially connected Agilent columns: Agilent Bio *SEC*-5 (5 μm particle size, 150 Å pore size) and Agilent Bio *SEC*-5 (5 μm particle size and 300 Å pore size) and a UV-detector. *In vitro* digests frozen at −80 °C were thawed and subjected to heat treatment (95 °C, 10 min) in a heat block to stop potential enzymatic activity. Then, samples were centrifuged for 10 min at 10,000 × g followed by filtering the supernatant through a syringe filter (0.45 μm, Minisart RC, Sartorius). Five μL of each sample was injected into the system and the absorbance was monitored at 214 nm for 60 min. The mobile phase was phosphate buffer (0.1 M; pH 7.50) with a flow of 0.625 mL/min. A commercial protein standard mix ranging from 1 to 670 kDa (AdvanceBio *SEC* 300 Å, Agilent) was used to create a calibration curve where the retention time of each peak was plotted against the logarithmic of its molecular size. Three independent injections were run for each digested sample and one injection for samples before digestion. To facilitate comparison between samples, peaks were grouped in the following categories: < 1 kDa, 1–5 kDa, 5–10 kDa, 10 – 670 kDa, and > 670 kDa. The relative proportion of each peak can be found in Supplementary

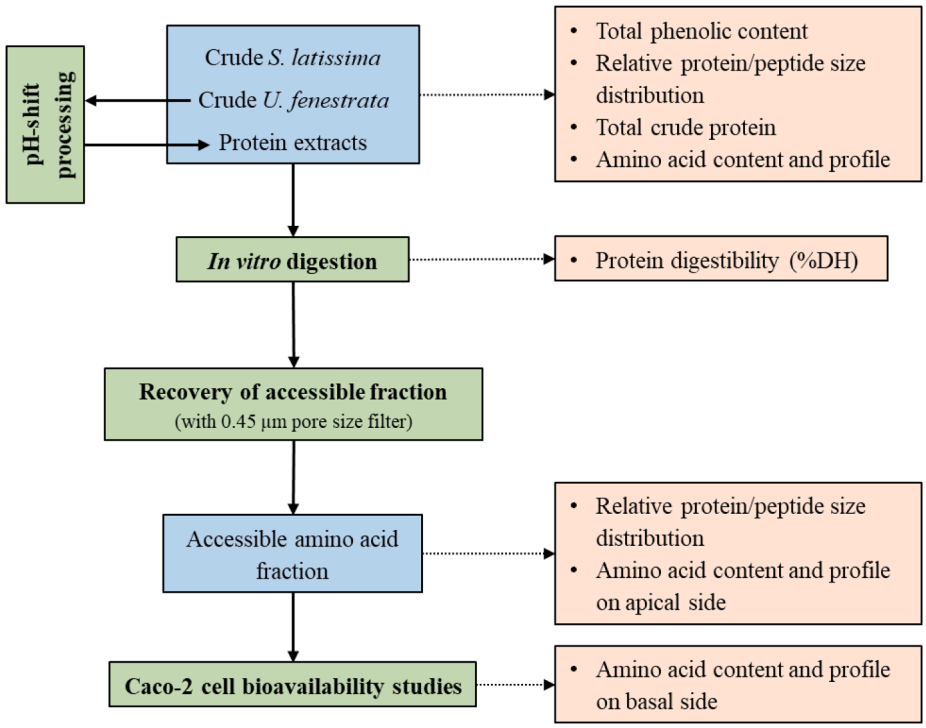


**Fig. 1.** Schematic picture over the samples (in blue), processes (in green), and analyses (in orange) used in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Material: Fig. S2. To assure that the centrifugation applied prior to the *SEC* analyses, as well as the heating used to inactivate digestive enzymes did not alter the protein/peptide profile; one digest (wet pH-shift extract) was systematically evaluated with *SEC* with and without heat treatment (95 °C, 10 min) and centrifugation applied. The results revealed no such changes (Supplementary Material: Fig. S3). Fig. 1 provides a schematic overview of this study.

### 2.11. Statistical analysis

To determine significant differences among samples concerning the different measures, one-way analysis of variance (ANOVA) was performed followed by Tukey's post hoc test for pairwise comparison. To compare significant differences in the amino acid profile of crude *U. fenestrata* and pH-shift protein extract thereof the Student's *t*-test was employed. The differences were considered statistically significant at $p < 0.05$. All statistical tests were conducted using SPSS Statistics software (version 26.0.0.0).

## 3. Results and discussion

### 3.1. Isolation of proteins using the pH-shift process

Total protein yield obtained in the pH-shift processing of *U. fenestrata* was 11.1 ± 3.7%, which is higher than other works that also extracted and precipitated proteins from *Ulva* sp. (Harrysson et al., 2018;

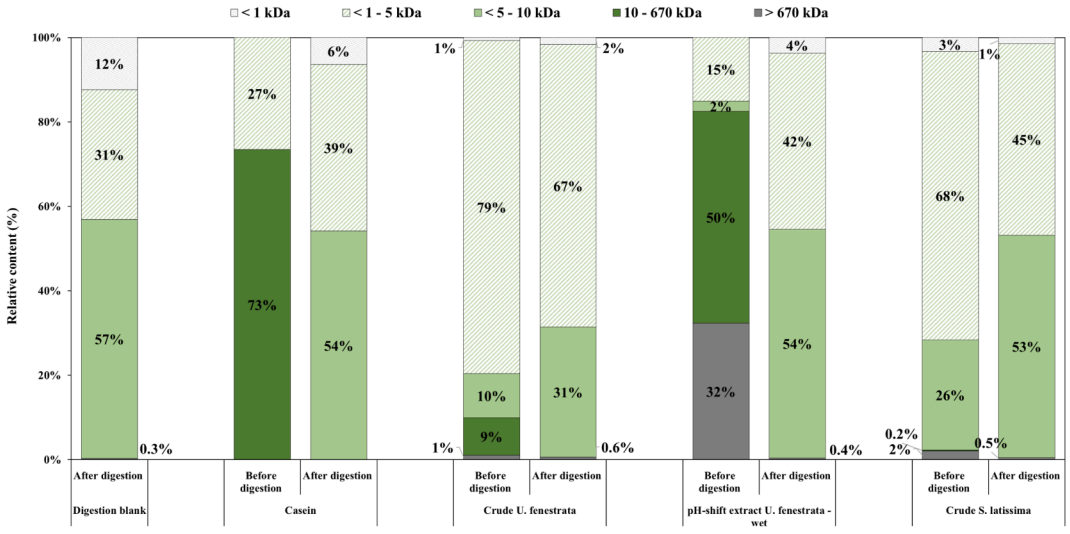


**Fig. 2.** Relative size distribution of the protein/peptides of casein, crude *U. fenestrata* and *S. latissima*, as well as pH-shift protein extract from *U. fenestrata* before and after *in vitro* digestion.

Magnusson et al., 2019; Robin et al., 2018). However, it is lower than in the study of Harrysson et al. (2019) (29%) who applied the same pH-shift protocol as used here. Possible explanations for such difference could be attributed to e.g. the content, profile, structure, and distribution of proteins in the biomasses used. The protein content in crude *Ulva lactuca* from Harrysson et al. (2019) was 12.8 ± 1.5% on DW basis, whereas the biomass in our study had a protein content of 16.5 ± 2.5%. Additionally, Harrysson et al. (2019) oven-dried the biomass after harvesting, whereas ours was frozen at −80 °C. As shown by Abdollahi et al. (2019), during the pH-shift processing of *S. latissima,* oven-dried biomass gave a yield of 20.1 ± 1.4%, which was significantly different when freezing the biomass at −80 °C (yield of 16.6 ± 1.2%).

The produced pH-shift extract contained 58.4 ± 5.7% protein on a DW basis, which represents a 3.5-fold up-concentration when compared to the protein content of the crude *U. fenestrata*. Previous works that also performed the pH-shift method concentrated *Ulva ohnoi, Ulva lactuca, Porphyra umbilicalis,* and *S. latissima* proteins up to 2.0-, 2.6-, 2.2, and 4.6-times, respectively (Abdollahi et al., 2019; Harrysson et al., 2018; Magnusson et al., 2019). Thus, our protein extraction protocol was in the mid to upper range when considering its efficiency to concentrate seaweed proteins.

From HP-*SEC* analyses primarily carried out to detect molecular size changes caused by the *in vitro* digestion (Fig. 2), a side-finding was that pH-shift processing of *U. fenestrata* increased by 43 percentage points the proportional area of a peak assigned to a polypeptide of about 49 kDa. Likewise, the proportional area of a peak eluted before the largest protein standard, bovine thyroglobulin (670 kDa), increased around 31 percentage points. This indicated that the pH-shift processing increased the relative proportion of high-molecular size protein fractions, which could be a result of both selective solubilization-precipitation of larger proteins and tentative cross-linking reactions of proteins/peptides.

### 3.2. Amino acid profile of seaweed biomass and pH-shift produced protein extracts

Table 1 presents the amino acid profile of crude *U. fenestrata* and protein extracts thereof. The pH-shift processing kept the relative content of valine, threonine, isoleucine, leucine, methionine, and phenylalanine, whilst increased the content of the remaining essential amino acids i.e. lysine and histidine ($p < 0.05$). Plant proteins can be deficient in certain essential amino acids when compared to the amino acid scoring pattern recommended by WHO/FAO/UNU (2007). However, our seaweed protein extract met the FAO/WHO requirements for adults. For children, the methionine requirement is given as the sum of sulfur amino acids, thus obstructing a comparison between this study and the reference values. Nonetheless, the relative content of the remaining amino acids complied with the FAO/WHO recommendations for this age group (WHO/FAO/UNU, 2007). Regarding the relative amount of essential amino acids (on a total amino acid basis), the produced pH-shift protein extract had similar values to previous pH-shift protein extracts from *U. lactuca* (42%) (Harrysson et al., 2018) and the casein used in this study (40%).

**Table 1**
Amino acid profile (g/100 g total amino acids) of crude *U. fenestrata* and pH-shift protein extract thereof. Essential amino acids are shown in bold.

| Amino acid | Crude *U. fenestrata* | pH-shift protein extract *U. fenestrate* |
|---|---|---|
| Glycine* | 5.07 ± 0.43 | 3.73 ± 0.02 |
| Alanine* | 7.65 ± 0.70 | 5.25 ± 0.02 |
| Serine* | 4.99 ± 0.26 | 4.19 ± <0.01 |
| Proline* | 6.17 ± 0.67 | 3.07 ± 0.15 |
| **Valine** | 6.47 ± 0.46 | 6.12 ± 0.05 |
| **Threonine** | 5.62 ± 0.46 | 5.73 ± 0.02 |
| **Isoleucine** | 4.05 ± 0.26 | 4.51 ± 0.01 |
| **Leucine** | 7.74 ± 0.64 | 7.77 ± 0.01 |
| Aspartic acid | 10.25 ± 0.81 | 9.55 ± 0.04 |
| **Lysine*** | 3.96 ± 0.03 | 5.45 ± 0.04 |
| Glutamic acid* | 12.13 ± 1.03 | 9.91 ± 0.03 |
| **Methionine** | 1.50 ± 0.11 | 1.66 ± 0.02 |
| **Histidine*** | 2.98 ± 0.10 | 4.15 ± 0.01 |
| **Phenylalanine** | 5.26 ± 0.29 | 5.29 ± 0.04 |
| Arginine* | 5.22 ± 0.09 | 5.69 ± 0.03 |
| Tyrosine* | 3.45 ± 0.03 | 4.34 ± 0.04 |
| TEAA | 37.58 | 40.68 |

EAA: total essential amino acids; * denotes significantly different values among samples ($p < 0.05$).

### 3.3. Protein digestibility - degree of protein hydrolysis, total phenolic content, and molecular size distribution

Protein digestibility as %DH is shown in Fig. 3. The %DH measured at the end of digestion was higher in the wet pH-shift protein extract ($p < 0.05$) than in crude *U. fenestrata* - 35.7 ± 2.1% vs 27.7 ± 2.6%. Also, crude *U. fenestrata*, as well as both the wet and freeze-dried protein extracts, were as digestible as casein (32.9 ± 1.6%). On the other hand, crude *S. latissima* presented a %DH of 16.9 ± 2.5%, which is 1.9- and 1.6-times lower than casein and crude *U. fenestrata,* respectively ($p < 0.05$). Additionally, when digesting a sample amount equal to 50% of the protein content of the original sample (pH-shift extract *Ulva* - wet), similar %DH values were obtained. This suggests that the digestive enzymes were not saturated with substrate, thus not limiting the %DH.

Before digestion, primary amines corresponded to 5.9 ± 0.3% and 2.7 ± 0.3% of all peptide bonds in crude *U. fenestrata* and pH-shift protein extracts, respectively (Fig. 3). The percentage difference between both groups was significant ($p < 0.05$) and probably suggests the removal of primary amines during pH-shift processing. The amine loss could be in the form of small peptides and/or free amino acids as these molecules can have higher solubilities than larger peptides/proteins, thus hampering their isoelectric precipitation. This hypothesis is corroborated by the *SEC* data of crude seaweed and protein extracts before and after pH-shift processing (Section 3.1).

Protein extracts from *Ulva* sp. produced by alkaline solubilization followed by dialysis and ion exchange purification (Kazir et al., 2019), hot alkaline solubilization and dialysis (Gajaria et al., 2017), and alkaline solubilization followed by precipitation with ammonium sulfate and dialysis (Wong & Cheung, 2001) have been reported to have a %DH equal to 86–89% of that for casein after *in vitro* digestions. In our study, there were no significant differences between %DH of the protein extracts and casein after completed digestion, although the ratio between the two in the mentioned order was 108%. The cited studies did not analyze the %DH of crude seaweed, thus making our study the first one to suggest that pH-shift based extraction of seaweed proteins can improve *in vitro* digestibility. As initially hypothesized, the removal of phenolics during the pH-shift processing could be the reason behind the improvement in digestibility. However, we found that pH-shift processing of *U. fenestrata* significantly concentrated the total phenolics from 0.93 ± 0.02 to 1.31 ± 0.16 mg phloroglucinol eq./g DW basis ($p < 0.05$); a finding that can be attributed to the reactivity of phenolic compounds with proteins (Bikker et al., 2016). Therefore, the higher %DH after pH-shift processing was likely due to mechanical disintegration of cell walls and/or removal of fibres. The role of fibre in digestibility of extracted seaweed protein has yet to be addressed. Nevertheless, studies on crude seaweed suggested that pre-treatment with cell-wall degrading enzymes increased protein digestibility of the resulting biomass, which was ascribed to the release of cell wall-bound protein or encapsulated protein in poorly digestible cell wall polysaccharides (Bikker et al., 2016; Mæhre, Jensen, & Eilertsen, 2016).

Crude *S. latissima* presented a total phenolic content of 8.51 ± 0.35 mg phloroglucinol eq./g DW basis – around 9.2-times more than *U. fenestrata* ($p < 0.05$). A study on 12 seaweed species revealed an inverse and non-linear correlation between total phenolic content and seaweed protein digestibility (Tibbetts, Milley, and Lall 2016). Thus, the high phenolic content of *S. latissima* may explain its low digestibility when compared to *U. fenestrata*. According to Tibbetts, Milley, and Lall

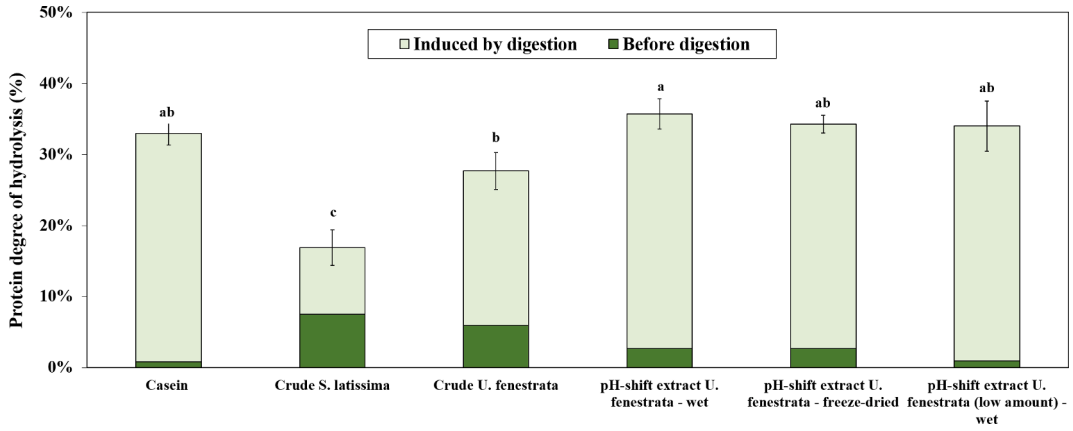


**Fig. 3.** Protein degree of hydrolysis (%DH) after *in vitro* digestion of casein, crude *U. fenestrata*. and *S. latissima* as well as pH-shift protein extracts from *U. fenestrata*. Lowercase letters indicate differences ($p < 0.05$) between samples. The dark green part shows the %DH present in samples already from start of the digestion. Note that standard deviations and lowercase letters correspond to the absolute %DH after digestion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2016), phenolics can decrease protein digestibility through two mechanisms: hydrogen binding and/or oxidation of proteins and amino acids, rendering them less available to enzymatic hydrolysis. The presence of alginate could also contribute to the lower digestibility since it can form gel matrices capable of entrapping proteins, thus limiting the action of the digestive proteases (Guo et al., 2020).

To get a more qualitative picture of the enzymatic cleavage of seaweed proteins, HP-*SEC* analyses were carried out before and after the *in vitro* digestion to unravel the relative molecular size distribution (Fig. 2). As expected from the %DH data, the amount of larger size proteins was reduced during the digestion in favor of smaller peptides. For instance, after digestion, the fraction 10–670 kDa was no longer detected in crude *S. latissima*, crude *U. fenestrata*, and pH-shift extract from *U. fenestrata*. Similarly, the > 670 kDa fraction of the latter sample appeared to be extensively cleaved to fragments between < 1 and 10 kDa. These findings further strengthen that seaweed protein - either in their crude form or after pH-shift extraction - can be hydrolyzed by mammalian proteases.

### 3.4. Amino acid accessibility - recovery of total amino acids after filtration of digests

Filtration of digests is described in the literature as a way to obtain the accessible fraction (Rubio & Clemente, 2009). This is in line with findings that cell monolayers can only transport free amino acids (Jochems, Garssen, Van Keulen, Masereeuw, & Jeurink, 2018) and peptides (Xu, Hong, Wu, & Yan, 2019), whereas the transport of amino acids/peptides/proteins embedded in large undigested or partly digested fragments remains unclear. As presented in Table 2, the digestion blank and casein showed the highest amount of accessible amino acids (83–85% of total), followed by the pH-shift protein extracts (72–73%) and the crude seaweeds (56–57%), having these latter samples significantly lower accessibility when compared to the others ($p < 0.05$). The gradual decrease in accessibility throughout the three groups could be related to the increasing amount of visible fragments as these remained in the non-accessible fraction i.e. retentate (Supplementary Material: Fig. S4).

### 3.5. Caco-2 cell transport studies

#### 3.5.1. Amount of individual amino acids on basal side after cell incubation

Fig. 4B depicts the amount of individual amino acids after 2 h of cell incubation in the basal side. The main amino acids present were glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, methionine, phenylalanine, and tyrosine, whereas aspartic acid, lysine, glutamic acid, histidine, and arginine were only quantifiable on the apical side (Fig. 4A). According to Wang and Li (2018), neutral peptides have higher transport rates than positively and negatively charged peptides, which could explain the results. Other works only observed a preference for isoleucine, leucine, tyrosine, and arginine in the Caco-2 protein thelial transport of amino acids (Goulart et al. 2014).

The production of wet pH-shift protein extract increased the basal side amounts of essential amino acids such as valine, threonine, isoleucine, leucine, and phenylalanine 1.71-, 1.52-, 1.62, 1.71-, and 1.80-fold, respectively ($p < 0.05$) when compared to the crude biomass. Likewise, higher levels of non-essential amino acids such as alanine and serine were also found in the basal medium originating from the wet pH-shift protein extract compared to crude *U. fenestrata* ($p < 0.05$). Increased basal amounts after pH-shift processing were probably due to the higher recovery of total amino acids after filtration resulting in more apical amino acids available for transport (Fig. 4B). Compared to casein, the wet pH-shift extract resulted in similar basal amounts of all bioavailable amino acids ($p > 0.05$) except for alanine (higher levels, $p < 0.05$) and proline and leucine (lower levels, $p < 0.05$).

The transport of amino acids from apical followed a proportional behavior since the total amount of basal amino acids decreased by half when half the amount of crude protein was subjected to *in vitro* digestion (Table 2). As transport of peptides across Caco-2 cell monolayers have been described by a saturation pattern (Wang & Li, 2017), working in linear ranges assures better estimations of bioavailability.

#### 3.5.2. Bioavailability

Table 2 shows the relative amino acid transport from the apical-to-basal side of *in vitro* digests samples. Amino acids from digested and filtered crude seaweeds and protein extracts from *U. fenestrata* were as bioavailable as casein. As the absolute amount of amino acids transported across the Caco-2 cell monolayers depends on both the accessibility and bioavailability, we suggest relating these two terms by multiplying them. The resulting parameter alongside protein digestibility and amino acid composition could give an additional dimension when assessing the nutritional quality of food proteins. In this work, such a parameter was higher in the pH-shift extracts than in crude *U. fenestrata*.

The digestion blank (i.e. digestive compounds without sample) presented the highest amino acid bioavailability probably because the amount of amino acids naturally secreted by the cells to the basal medium ($0.010 \pm 0.001$ mg) was not subtracted from the calculations (Eq. (4)). If doing so, the digestion blank would have a similar bioavailability to the remaining samples ($p > 0.05$). However, the chemical and structural properties of each peptide regulate their apical-to-basolateral

**Table 2**
Accessibility (i.e. recovery after filtration) and bioavailability of the *in vitro* digests. Total crude protein before digestion are also shown together with total amino acids before and after digestion, of the filtered digests, and of the apical and basolateral sides.

| | | Cell control | Digestion blank | Casein | Crude *S. latissima* | Crude *U. fenestrata* | pH-shift extract *U. fenestrata* – wet | pH-shift extract *U. fenestrata* – freeze-dried | pH-shift extract *U. fenestrata* (low amount) – wet |
|---|---|---|---|---|---|---|---|---|---|
| | Total crude protein from start (mg)[1] | – | 0 | $67.2 \pm 0.4^{a}$ | $32.9 \pm 0.5^{b}$ | $67.2 \pm 1.9^{a}$ | $67.2 \pm 0.8^{a}$ | $67.4 \pm 0.8^{a}$ | $33.6 \pm 0.4^{b}$ |
| Total amino acids | Before digestion (mg) | – | 0 | $62.9 \pm 4.6^{a}$ | $29.4 \pm 0.4^{b}$ | $51.9 \pm 7.9^{a}$ | $65.2 \pm 6.4^{a}$ | $65.4 \pm 6.4^{a}$ | $32.6 \pm 3.2^{b}$ |
| | Digest at the end of digestion (mg)[2] | – | $15.8 \pm 0.7$ | $78.7 \pm 4.7$ | $45.2 \pm 0.8$ | $67.7 \pm 7.9$ | $81.0 \pm 6.4$ | $81.2 \pm 6.4$ | $48.4 \pm 3.3$ |
| | Filtered digest (mg) | – | $13.3 \pm 0.3^{a}$ | $65.5 \pm 1.9^{b}$ | $25.4 \pm 1.1^{d}$ | $38.5 \pm 2.8^{c}$ | $59.3 \pm 3.8^{b}$ | $58.2 \pm 3.3^{b}$ | $35.3 \pm 2.1^{c}$ |
| | On apical side from 250 µL filtered digest (mg) | – | $0.22 \pm 0.01^{a}$ | $1.09 \pm 0.03^{b}$ | $0.42 \pm 0.02^{d}$ | $0.64 \pm 0.05^{c}$ | $0.99 \pm 0.06^{b}$ | $0.97 \pm 0.07^{b}$ | $0.59 \pm 0.03^{c}$ |
| | On basolateral side after incubation (mg) | $0.010 \pm 0.001^{a}$ | $0.029 \pm 0.004^{ab}$ | $0.083 \pm 0.019^{c}$ | $0.036 \pm 0.007^{abc}$ | $0.046 \pm 0.008^{abc}$ | $0.075 \pm 0.023^{bc}$ | $0.062 \pm 0.020^{bc}$ | $0.045 \pm 0.004^{abc}$ |
| | Accessibility, i.e. amino acid yield in filtrate (%)[3] | – | $84.7 \pm 1.7^{a}$ | $83.3 \pm 2.4^{ab}$ | $56.3 \pm 2.4^{c}$ | $56.9 \pm 4.1^{c}$ | $73.2 \pm 4.7^{ab}$ | $71.9 \pm 4.1^{b}$ | $73.0 \pm 3.9^{ab}$ |
| | Bioavailability (%)[4] | – | $13.2 \pm 1.9^{a}$ | $7.6 \pm 1.6^{b}$ | $8.5 \pm 1.2^{ab}$ | $7.3 \pm 1.3^{b}$ | $7.5 \pm 1.8^{b}$ | $6.4 \pm 2.0^{b}$ | $7.8 \pm 1.0^{b}$ |

Within the same line, different lowercase letters mean differences between values ($p < 0.05$).
[1]Analyzed as Total N × conversion factor. These factors were 5.00 for crude seaweeds and 6.25 for the pH-shift protein extracts and casein.
[2][Total amino acids before digestion + Total amino acids from digestive compounds].
[3][Total amino acids of filtered digest]/[Total amino acids before digestion + Total amino acids from digestive compounds] × 100.
[4][Total amino acids on basolateral side after incubation / Total amino acids added on apical side] × 100.

transport (Xu, Hong, Wu, & Yan, 2019), thus we choose not to deduct the contribution of the cell and digestion blanks as their bioavailability could be influenced by sample-derived peptides.

Regarding individual amino acids, serine, proline, and methionine were less bioavailable ($p < 0.05$) compared to glycine, alanine, threonine, isoleucine, leucine, phenylalanine, and tyrosine (Fig. 4C). Moreover, the bioavailability of each amino acid was similar regardless if it was crude seaweed or pH-shift protein extracts.

To the best of our knowledge, no earlier work has explored the Caco-2 cell amino acid bioavailability of seaweed protein extracts, though a few other vegetarian protein sources have been evaluated. Rubio and Clemente (2009) studied amino acid bioavailability from chickpea and lupin protein isolates and found casein to have higher bioavailability on the following amino acids: aspartic acid, glycine, histidine, arginine, alanine, tyrosine, phenylalanine, and lysine; whereas similar bioavailability was found for glutamic acid, serine, threonine proline, valine, isoleucine, and leucine. This differs from our results where crude *U. fenestrata* and extracts thereof had the same amino acid bioavailability as casein. Whether the higher purity of their isolates (77–87% of protein on a DW basis) compared to our protein extracts (58% protein on a DW basis) affected the relation to casein needs to be further studied. Although not evaluated in full detail, we know that the remaining 42% of protein extract from *Ulva* spp. comprises e.g. carbohydrates, minerals, fatty acids, and chlorophylls (Harrysson et al., 2018). As stated above, carbohydrates/fibres and phenolics can indeed reduce the nutritional quality of seaweed proteins in terms of its digestibility (Özkan Gülzari et al., 2019; Tibbetts et al., 2016), but, as far as we know, nothing is described regarding the effect of minerals, non-phenolic pigments, and fatty acids on the amino acid bioavailability.

Some studies have addressed bioavailability of hydrolyzed protein from soy, fish, collagen, and whey (Feng & Betti, 2017; Goulart et al., 2014; Mcgraw et al., 2014; Samaranayaka, Kitts, & Li-Chan, 2010). However, these works did not use casein as a reference protein, which hinders comparisons between results. In one of the listed studies, bioavailability was measured based on amino group content (Samaranayaka et al., 2010), which is not directly comparable to amino acid bioavailability.

Despite being a good screening tool to mimic certain *in vivo* processes, the Caco-2 cell model lacks intestinal peristalsis motions, which are known to improve the mechanical degradation of nutrients (Welcome, 2018). Moreover, it is only constituted of enterocytes, lacking other epithelial cells like mucus-producing cells. The mucus secreted by these cells can influence nutrient absorption and protect the epithelium (Jochems et al., 2018). Therefore, animal studies are warranted to confirm the findings presented here.

It has been reported that short-chain peptides (<500 Da) present higher cell bioavailability than relatively long-chain peptides (<2000 Da) (Feng & Betti, 2017; Wang & Li, 2017). Therefore, as samples in this study presented similar bioavailability values (Table 2), we hypothesized they would have similar protein/peptide size profiles after *in vitro* digestion. To test this assumption, HP-*SEC* was applied to digests of each sample type (Fig. 2). The relative percentage of peptides < 1 kDa was significantly higher in digested casein than in digested crude seaweeds and digested pH-shift extract from *U. fenestrata* ($p < 0.05$). Regarding fraction 1–5 kDa, its relative percentage was significantly higher in digested crude *U. fenestrata* when compared to the other samples ($p < 0.05$). An opposite trend was observed for the fraction 5–10 kDa, meaning that digested crude *U. fenestrata* presented the lowest percentage for this peptide fraction ($p < 0.05$). Overall, we detected significant variations between samples, so it was not possible to disclose a clear relationship between bioavailability and protein/peptide relative size distribution after *in vitro* digestion. According to Xu, Hong, Wu, and Yan (2019), bioavailability can be influenced by other properties than just size, such as hydrophobicity, amino acid sequence (e.g., groups at N- and C-terminus), and side-chain flexibility. Moreover, the digests were evaluated before their addition to the Caco-2 monolayers, so we did not

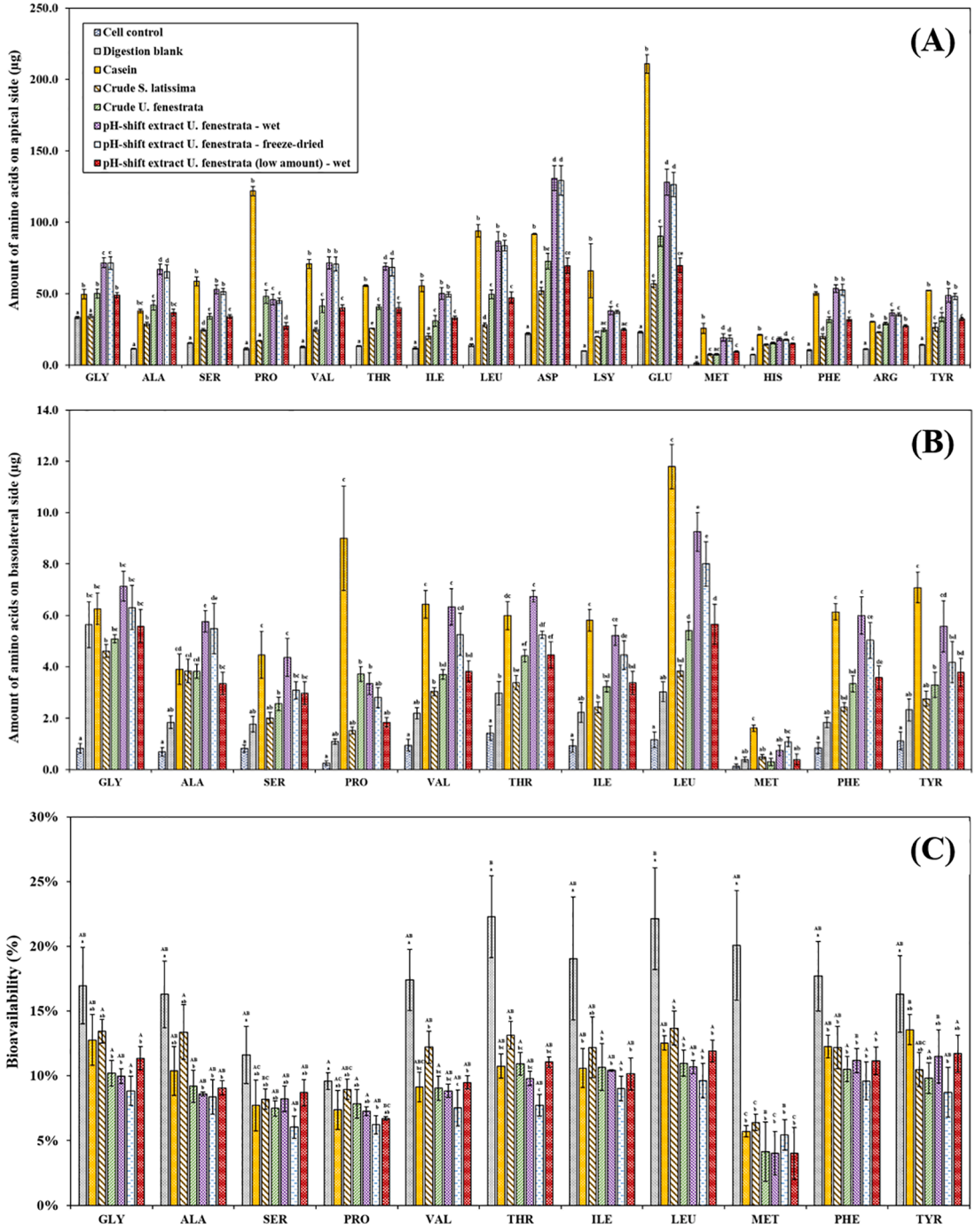


**Fig. 4.** Caco-2 cell transport studies: (A) amount of each amino acid on the apical side right before incubation [µg]; (B) amount of each amino acid on basal side after 2 h incubation [µg]; (C) amino acid bioavailability [%]. Lowercase letters in (A), (B) and (C) indicate differences between samples for a given amino acid ($p < 0.05$). Upper case letters in (C) indicate differences between amino acids ($p < 0.05$).

capture the tentative digestion of protein material into smaller peptides and free amino acids, which can be carried out by brush-border and cytosolic peptidases (Xu, Hong, Wu, & Yan, 2019). Taking apical samples after the 2 h incubation would only show the residual peptides not transported through the monolayers why a complete picture of the tentative Caco-2 cell-induced proteolysis is difficult to capture.

## 4. Conclusion

The main aim of this study was to assess the influence of a protein extraction method on the digestibility, accessibility, and cell bioavailability of *U. fenestrata* proteins. As expected, the pH-shift processing concentrated the proteins from *U. fenestrata* and yielded a polypeptide profile with higher molecular weight profile - probably due to selective solubilization-precipitation of larger proteins and cross-linking reactions of proteins/peptides. Moreover, the pH-shift processing significantly improved the *in vitro* protein digestibility and amino acid accessibility of *U. fenestrata* to values close to those of casein. The increase in digestibility was most likely not related to the removal of phenolics as these antinutrients were actually concentrated during the pH-shift processing.

Regarding Caco-2 cell bioavailability, the accessible amino acid

fraction of all samples was as bioavailable as casein. However, based on the higher amino acid accessibility resulting from pH-shift processing, the total amounts of nearly all amino acids transported across the cells was higher in pH-shift protein extracts from *U. fenestrata*. The analysis of the protein/peptide molecular size distribution of the samples after *in vitro* digestion did not disclose a clear association with bioavailability. Therefore, other factors such as hydrophobicity, amino acid sequence, side-chain flexibility, or cell-induced proteolysis may explain the similar bioavailabilities.

Overall, the pH-shift processing can be a suitable method to use when exploring seaweed as a novel vegan protein source since it can concentrate its protein whilst improving its digestibility after *in vitro* digestion. However, *in vivo* experiments (e.g. rat models) are warranted to validate these findings as well as to further investigate the interlinks between pH-shift processing, accessibility, and bioavailability.

## CRediT authorship contribution statement

**João P. Trigo:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Niklas Engström:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - review & editing. **Sophie Steinhagen:** Formal analysis, Investigation, Writing - review & editing. **Louise Juul:** Formal analysis, Investigation, Data curation, Writing - review & editing. **Hanna Harrysson:** Resources, Funding acquisition. **Gunilla B. Toth:** Resources, Writing - review & editing, Funding acquisition. **Henrik Pavia:** Resources, Funding acquisition. **Nathalie Scheers:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Funding acquisition. **Ingrid Undeland:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## 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.foodchem.2021.129683.

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