Article

Marine Macroalgae in Rabbit Nutrition: *In Vitro* Digestibility, Caecal Fermentability, and Microbial Inhibitory Activity of Seven Macroalgae Species from Galicia (NW Spain)

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Abstract: The limitation on the prophylactic use of antibiotics in animal feed in Europe has critically challenged the rabbit meat industry, which urgently needs to find solutions. A feasible alternative could be using macroalgae in the diet to improve the gut health. This research studied seven species of marine macroalgae in four formats (dehydrated, enzymatically hydrolyzed, aqueous extract, and aqueous extract of hydrolyzed macroalgae) in order to select the most promising ones for their use in rabbit feed. Chemical composition, *in vitro* digestibility, *in vitro* caecal gas, total volatile fatty acid (VFA) production, and minimal inhibitory concentrations (MIC) against common pathogens were studied. All *S. latissima* products showed high caecal fermentability and VFA production, especially in both types of extracts. The *H. elongata* aqueous extract was remarkable due to its high *in vitro* butyrate production, which can be of great interest for improving gut health. The MIC results did not indicate any clear inhibition of the pathogens tested. The macroalgae tested appear to have a potentially prebiotic effect, rather than a direct antimicrobial activity. However, these results must be confirmed *in vivo*, in order to observe the real benefits of feeding macroalgae during the rabbit weaning period.

Keywords: *in vitro* digestibility and gas production; marine macroalgae; microbial inhibitory activity; rabbit; nutrition

1. Introduction

Rabbit meat is a valuable niche product in Mediterranean countries such as Spain, France, and Italy, and the EU is the second biggest producer of this foodstuff in the world [1]. This meat is known for its beneficial nutritional properties—as a source of high-quality protein (CP), B group vitamins, and healthy fatty acids—and also for its characteristic taste and tenderness [1–5].

In Europe, the rabbit industry is undergoing a critical period because of the gradual reduction in the consumption of this type of meat and the many structural weaknesses in the production system [1,6]. Moreover, the limitation on the prophylactic use of antibiotics in animal production produced in Europe in the last years led to an increase in rabbit mortality in many farms, often making production almost economically unsustainable [7,8]. In this respect, the main challenge in rabbit production is to maintain gut health in the rabbits during the post-weaning period [9–11], when the digestive tract is very vulnerable and susceptible to the proliferation of bacterial pathogens (e.g., *Clostridium perfringens* and *Escherichia coli*) and parasitic protozoa (e.g., coccidians) [12]. In this context, alternatives to antibiotics that improve the gut health and resilience of rabbits to pathogens are required [6], and dietary strategies are especially key in the post-weaning period [13,14]. The addition of marine macroalgae to rabbit diets is a very promising alternative approach, providing numerous benefits that have already been demonstrated in other animal production systems (for reviews, see [6,15–18]).

Marine macroalgae are classified into brown (Phaeophyta), green (Chlorophyta), and red macroalgae (Rhodophyta) [19,20]. Macroalgae have a particular nutritional make-up that is very different from that of most animal feeds, although the composition varies widely across species [21]. They contain high levels of minerals and vitamins, moderate or low levels of fat, although they are usually rich in polyunsaturated fatty acids (PUFAs), polyphenolic compounds, and a variable protein level [22–24]. In addition, macroalgae show great potential as animal feed due to their high contents of dietary fibre (25–75% of dry matter (DM)) [25,26] that includes insoluble and soluble fibre. The insoluble fibre, which is composed of hemicellulose, cellulose, and lignin, is poorly fermented [25,26]. The soluble fibre is mainly composed of carbohydrates such as alginates, laminarins, and fucoidans in brown macroalgae, of ulvans and sulphated galactans in green macroalgae, and of agars and carrageenans in red macroalgae [27–29]. These polysaccharides are not generally digested in the small intestine of farm animals and can therefore be either partly or fully fermented in the large intestine. Fermentation of soluble fibre by gut microbiota produces different gases and metabolites like lactic acid and volatile fatty acids (VFA), which modulate the gut environment [6,30]. It is therefore important to determine how macroalgae are fermented, because this process determines their potential prebiotic effects [31–33]. In addition, some macroalgae components may have antimicrobial, immunomodulatory, anti-inflammatory, and antioxidant properties, and it has been suggested that including them in livestock diets could potentially improve meat quality [22,23]. In summary, feeding macroalgae could improve gut health in farm animals and therefore promote nutrient absorption, animal growth, disease protection, and animal welfare [20,34]. Moreover, the inclusion of macroalgae in animal feed has many environmentally related benefits (for reviews, see [6,20,34]), as their production does not require fresh water, fertilization, or arable land [35] and it provides many ecosystem services [36].

The Atlantic coast of Galicia (north-western Spain) is a privileged enclave for macroalgae production, as it hosts more than 700 species with very high production rates [37,38]. Numerous companies in the region either harvest or cultivate macroalgae in a sustainable manner for human consumption [39], and during processing each company accumulates more than 40,000 kg of macroalgal discards every year that cannot be marketed. Recycling these discards in animal feeding, following a circular economy approach, would add value to the macroalgae industry and provide a high-quality ingredient for animal feed [37,40], including rabbit feed [6]. This approach would reinforce both the macroalgae and rabbit production sectors, and could also be used as a marketing strategy to increase rabbit meat consumption by environmentally responsible consumers [40].

Despite the aforementioned benefits, experience regarding the inclusion of macroalgae in animal feed remains scarce, especially in the case of rabbit production. In addition, the great diversity of marine macroalgae makes correct characterization of their properties necessary before their inclusion in animal feed. The objective of this study was therefore to characterize 19 products obtained from seven macroalgae species from the Galician coast, by analyzing

their chemical composition, *in vitro* digestibility, and caecal fermentation, and minimum inhibitory concentrations (MICs), in order to assess their potential benefits in rabbit health. The ultimate aim was to select the most promising macroalgae for further *in vivo* evaluation.

2. Materials and Methods

Data collection was carried out according to Directive 2010/63/EU on the protection of animals used for scientific purposes, and the trial complied with the Spanish legislation on animal care (RD 53/2013). The procedures were approved by the Bioethics Committee of the Universidad Politécnica de Madrid, Spain (protocol code 2021-002, approved 24 February 2021).

2.1. Macroalgae Products

A total of 19 samples of macroalgae products were tested, including 7 species and 4 different types of macroalgae products. All samples were provided by Porto-Muiños S.L. (Cerceda, La Coruña, Spain) and were sustainably harvested or cultivated on the Atlantic coast of Galicia (NW Spain).

The macroalgae species were selected according to different criteria (Table 1). Some were chosen because they are either already cultivated in Galicia (*Saccharina latissima*) or potentially cultivable in the short term (*Himanthalia elongata*, *Undaria pinnatifida*, and *Ulva* spp.), and therefore their long-term production is guaranteed without relying on harvesting of wild populations [39,41]. Another criterion was that the industrial processing of the macroalgae produces discards that could be used for animal feed, thereby adding value to these industries and promoting a circular-economy strategy [6,40]. Other macroalgae like *Fucus vesiculosus* and *Mastocarpus stellatus* are highly abundant, but have little commercial value, and their use in animal feed could improve their market value. *Himanthalia elongata* has already been identified as a valuable ingredient for livestock diets [23] and also has potential benefits on human health [42]. Finally, *Ulva* spp. can improve gut health and productive performance of rabbits [24,43].

Table 1. Classification, selection criteria, and type of product tested for each macroalgae.

Name	Group	Selection Criteria	Type of Sample Tested
Saccharina latissima	Sugar kelp	Brown	Cultivated. Long-term future. Produces discards	- Dehydrated - Hydrolyzed - Aqueous extract - Hydrolyzed aqueous extract
Fucus vesiculosus	Fucus	Brown	Harvested. Abundant and underexploited	- Dehydrated - Hydrolyzed - Hydrolyzed aqueous extract
Himanthalia elongata	Sea spaghetti	Brown	Harvested, potentially cultivable. Produces discards. Proven benefits	- Dehydrated - Hydrolyzed - Aqueous extract
Undaria pinnatifida	Wakame	Brown	Harvested, potentially cultivable. Long-term future. Produces discards	- Dehydrated - Hydrolyzed - Aqueous extract
Laminaria ochroleuca	Kombu	Brown	Harvested. Produces discards	- Hydrolyzed - Hydrolyzed aqueous extract
Ulva spp.	Sea lettuce	Green	Harvested, potentially cultivable. Long-term future. Very abundant. Produces discards. Proven benefits	- Dehydrated - Hydrolyzed - Hydrolyzed aqueous extract
Mastocarpus stellatus	Star shaped moss	Red	Harvested. Very abundant	- Hydrolyzed aqueous extract

The selected macroalgae were tested in four different formats (Table 1) obtained after following various industrial processes: dehydrated, enzymatically hydrolyzed, aqueous extract, and aqueous extract of hydrolyzed macroalgae. The aqueous extracts were prepared with the aim of obtaining the compounds of interest (mainly soluble polysaccharides). Macroalgae are commonly dried to extend their shelf life, and the dehydrated macroalgae tested in this study were obtained by drying the samples at low temperature (<40 °C) for 4–5 days before grinding them in a micro-grinding mill (Komodin K-160 P, Lleal, Granollers, Spain) that rendered powder samples. The selected macroalgae were also subjected to enzymatic hydrolysis to improve both carbohydrates and protein digestibility. To this end, the samples were incubated for 24 h in a water solution (1:15 macroalgae:water; in weight) containing 200 μL/100 mL solution (pH 6, 50 °C) of the enzyme Pectinex Ultra tropical (Novozymes, Bagsvaerd, Denmark), and 200 μL/100 mL solution (pH 8, 55 °C) of the enzyme Alcalase (Sigma Aldrich, Darmstadt, Germany). The enzymes were inactivated by raising the temperature to 90 °C for 15 min. Then, the hydrolyzed macroalgae were lyophilized.

For the aqueous extraction of the soluble compounds of macroalgae, samples were mixed with water (1:30 m:v) and subjected to autohydrolysis in a Parr pressure reactor operating in a non-isothermal mode up to 160 °C and 110 psi. The supernatant was then collected and dried with a spray dryer (Büchi B-290, Flawil, Switzerland) equipped with a standard cyclone (1.5 mm nozzle). The operating settings were 115 °C inlet temperature, 4 mL/min (pump at 15%) feed solution flow rate, 1050 L/h atomization air flow rate, and 4.1 bar pressure. Finally, the aqueous extract of the enzymatically hydrolyzed macroalgae was obtained, as the enzymatic hydrolysis breaks the cell walls and release compounds of interest. Samples were mixed with water (1:10 ratio) containing the enzyme Celluclast (Novozymes, Bagsvaerd, Denmark) at 4%, and the mixture was incubated at 50 °C for 6 h. The enzyme was inactivated by raising the temperature to 90 °C for 15 min. The supernatant was then dried and ground as commented before. Different products were obtained for each macroalgae species, depending on its characteristics and commercial interest.

2.2. In Vitro Digestibility

The *in vitro* ileal and faecal digestibility of dry matter (DM) and the faecal digestibility of crude protein (CP) were determined in all samples as previously described [44,45], using the adapted Ankom bags method [46]. Briefly, 0.5 g of each sample was weighed and placed in an Ankom® filter bag (Ankom Technology, Fairport, NY, USA). Bags were introduced in a Daisy[II] Incubator jar and incubated first with a HCl + pepsin solution (0.25 g pepsin/g sample, pepsin from porcine gastric mucosa P7000-100G, Sigma, Darmstadt, Germany) for 2 h (step 1: stomach), then with a pancreatin solution (1 g pancreatin/g sample, pancreatin from porcine pancreas P7545-100G, Sigma, Darmstadt, Germany) for 3 h 30 min (step 2: small intestine), and finally with Viscozyme for 16 h (step 3: caecum). The weight of the residues obtained in steps 2 and 3 were used to determine the *in vitro* ileal (ivIDMd) and faecal DM (ivFDMd) digestibility, respectively. The faecal digestibility of CP (ivFCPd) was determined by analyzing the nitrogen (N) content in the final residue obtained after step 3. Sugar beet pulp (SBP) and untreated cereal straw were selected as reference samples for highly and poorly fermentable sources of fibre, respectively, and were subjected to the *in vitro* procedures described previously.

2.3. Estimation of Nutritional Value

The nutritional value of the samples was estimated from the chemical composition and the ivFDMd, as described for rabbit feed [47]. The corresponding equations are generally used for animal feed but not for individual ingredients, and the estimates obtained are therefore only indicative, although they helped in ranking the different macroalgae prod-

ucts. The following equations [47] were used to estimate the apparent faecal digestibility of DM (DMd) and of gross energy (GEd), and the digestible energy content (DE):

$$DMd = -0.019 + 0.98 \times (ivFDMd/100)$$

$$GEd = -0.003 + 0.95 \times (ivFDMd/100)$$

$$DE (MJ/kg DM) = 1.63 + 15.0 \times (ivFDMd/100).$$

2.4. In Vitro Caecal Fermentation and VFA Production

The potential fermentability of all samples in the caecum was assessed by measuring the *in vitro* gas production after the incubation of samples with caecal inoculum from rabbits as described by Abad-Guamán et al. [48]. The insoluble residues obtained after the 2-step *in vitro* DM digestibility procedure of the macroalgae products were also incubated *in vitro* when their ivIDMd was below 93%, as there was not enough residue for samples having greater ivIDMd values. Samples (200 mg DM) were accurately weighted into 60 mL fermentation vials.

A total of four unsexed crossbred hybrid rabbits (New Zealand White × Californian, V × R line from UPV, Valencia, Spain) weaned at 30 d of age and fed a standard diet (16.1% CP, 42.1% total dietary fibre (TDF), 32.6% neutral detergent fibre (NDF), 9.51% soluble fibre (SF)) were slaughtered by head concussion at 67 days of age and 2.3 ± 0.03 kg body weight, which is the usual market weight in Spain. Consequently, there were 4 replicates like in a previous study [48]. The caeca of the 4 rabbits were immediately separated, 8 g of caecal digesta of each rabbit was carefully weighted and separately mixed with 800 mL of Goering and Van Soest buffer solution [49], and the mixtures were homogenized for 1 min with a domestic blender. Twenty mL of the mixture was immediately added to each vial with the aid of a peristaltic pump (Watson-Marlow 520UIP31; Watson-Marlow Fluid Technology Group, Cornwall, UK). The vials were sealed with rubber stoppers and incubated at 39 °C. Gas production was measured with a pressure transducer (Wide Range Pressure Meter; Sper Scientific LTD, Scottsdale, AZ, USA) and a plastic syringe at 3, 8, 12, 24, 30, 36, 48, 58, 75, 103, and 119 h. After each measurement, the gas produced was liberated.

After 24 h of incubation, 1 mL of the content of each vial was collected by using an insulin syringe and mixed with 80 μL of 10% H_2SO_4 to stop fermentation. This sample was used to analyze VFA production in the samples that yielded at 48 h of incubation significantly more gas production than the untreated cereal straw used as reference (gas production > 45 mL/g DM). Samples were processed as described by Ocassio-Vega et al. [50] and VFA concentration was measured by gas chromatography [51] using a Pelkin Elmer Autosystem XL gas chromatograph (Perkin Elmer Inc., Shelton, CT, USA) equipped with an automatic injector, detector flame ionization, and a semi-capillary column (TR-FFAP 30 m × 0.53 mm × 1 μm; Supelco, Barcelona, Spain).

Each sample (19 macroalgae products + 12 insoluble residues of 2-step *in vitro* digestibility + 2 reference ingredients) were incubated with each of the 4 caecal inocula to obtain 4 replicates per sample, making a total of 132 vials with substrate. In addition, 8 vials with no substrate (blanks; 2/inoculum) were incubated to correct the amount of gas and VFA for endogenous production at each measurement time.

2.5. Minimal Inhibitory Concentrations (MIC)

The antimicrobial potential of the 19 samples of macroalgae products was tested with 6 strains of aerobic pathogenic bacteria (strains acquired from the CECT (Spanish Type Culture Collection, University of Valencia): *Escherichia coli* (CECT 727 and CETC 434), *Salmonella typhimurium* (CECT 4594), *Salmonella enteritidis* (CECT 4300), *Listeria monocytogenes* (CECT 4032) and *Pseudomonas aeruginosa* (CECT 108). The antimicrobial potential was also tested with 6 strains of anaerobic pathogenic bacteria (provided by the CECT): *Clostridium perfringens* (CECT 376, CECT 486 and CECT 563) and *Clostridium difficile* (CECT

531, CECT 9136 and CECT 9137). Additionally, 2 aerobic pathogenic bacteria obtained from weaned rabbits were tested: *Escherichia coli* R1 and R2 (provided by the *Escherichia coli* Reference Laboratory (LREC, University of Santiago de Compostela, Spain).

The lyophilized strains of bacteria were reconstituted following the CECT guidelines. Viability, multiplication capacity, and identification were confirmed on selective/differential culture media and under temperature, time, and oxygen conditions suitable for each genus: VRBL-Violet Red Bile Glucose agar for *E. coli*, SM2-chromID Salmonella chromogenic agar, ALOA-chromogenic agar for *Listeria* spp., CFC agar for *Pseudomonas* spp., and Clostridium Reinforced agar for clostridia. The strains were then frozen in tubes with cryoballs in phosphate buffered saline (PBS) ($-20\ ^\circ$C) until use.

The highest concentration of macroalgae products tested in this study (8.2 mg/mL) was selected in order to replicate the *in vivo* conditions when macroalgae are used in animal feed, with levels up to 2.5% of macroalgae in the diet [21,23,32]. A dilution factor of about 3.28 was assumed due to water consumption, as in rabbits it may be around 1.5 g water/g feed, although this may be higher depending on the type of dietary fibre. The potential beneficial effects of macroalgae in animal/rabbit nutrition were observed at around 1–2% of the diet [21,23,32].

2.5.1. Aerobic Microorganisms: Broth Dilution Method

The method used to assess the antimicrobial potential of the macroalgae products with the aerobic strains was serial microdilution in polystyrene microplates, following the recommendations of the Clinical and Laboratory Standards Institute [52].

Aliquots (65 mg) of each sample were weighed and dissolved in 2 mL of 1% dimethyl sulfoxide (DMSO), to achieve an initial concentration of 32.5 mg/mL DMSO. In order to remove any impurities present, the diluted samples were filtered through a 0.22 μm filter into sterile tubes. From this initial extract, serial twofold dilutions were made in culture medium (Mueller–Hinton Broth), which is ideal for growth of these bacteria in microplates. Therefore, the concentrations tested ranged from 0.03 to 8.2 mg/mL (0.03, 0.06, 0.13, 0.26, 0.51, 1.02, 2.05, 4.10, and 8.2 mg/mL).

The 8 strains of aerobic bacteria tested were diluted in Mueller–Hinton Broth to a concentration of 10^8 cfu/mL (corresponding to the 0.5 MacFarland standard). For the assay, a 96-well microplate was used for each macroalgae sample. Aliquots (50 μL) of the corresponding macroalgae product dilution and of the bacterial solution were dispensed in each individual well. The DMSO (1:10) was used as a negative control and lactic acid (40%) as a positive control. Duplicate microplates were assessed for each macroalgae product. Microplates were incubated at 37 $^\circ$C for 24 h before interpretation of results. The MIC was determined as the lowest concentration of sample solution that completely inhibited growth of the bacteria in the microdilution wells.

2.5.2. Anaerobic Microorganisms: Agar Dilution Method

For the anaerobic bacterial strains, the method used to evaluate the antimicrobial potential of the macroalgae samples was the agar dilution method, following the guidelines of the Clinical and Laboratory Standards Institute [53].

Macroalgae products were tested at 2 different concentrations (6 and 8.2 mg/mL). The samples were weighed (0.32 and 0.20 g, respectively) and dissolved in 2 mL of DMSO/EtOH 50%; and then diluted in 18 mL of Clostridium reinforced agar at 50 $^\circ$C. Each preparation was thoroughly mixed, transferred to a Petri plate (19 samples × 2 concentrations), and left to solidify completely. Aliquots (2 μL) of each of the 6 Clostridia strains were inoculated in each plate, in duplicate (10^8 cfu/mL, 0.5 McFarland ABS). Lactic acid (40%) was used as positive control, and an identical plate without macroalgae products was used as negative control with the same Clostridia strains. The plates were incubated in anaerobiosis boxes at 37 $^\circ$C for 48 h. Duplicate plates were assessed for each macroalgae product and each concentration tested. The results were considered negative if the bacteria were able to

grow in the agar containing the macroalgae products and positive if bacterial proliferation was inhibited.

2.6. Chemical Composition

The AOAC (2000) methods were used to determine dry matter (method 934.01), ash (method 942.05), nitrogen (N; method 968.06), TDF (985.29), acid detergent fibre (ADF), and acid detergent lignin (ADL) in the macroalgae products and reference ingredients (973.18, ref. [54]). The NDF was determined using the filter bag system [55] (Ankom Technology, New York, NY, USA), with thermo-stable amylase and without any sodium sulphite added. The NDF was corrected for the ash and protein content, as indicated for total dietary fibre, while ADF and ADL were only corrected for the ash content of the ADL residue. The SF was calculated by difference, as TDF–NDF. Crude protein was calculated as N × 5 [56]. The difference 100-ash-CP-TDF was calculated to estimate the potential dietary fibre not retained in TDF (RES), assuming low levels (<10%) of sugars/oligosaccharides, starch, and ether extract in the samples.

2.7. Statistical Analysis

Gas production values measured at each time and VFA values at 24 h were corrected for the amount of gas and VFA, respectively, produced in the corresponding blanks to discount the endogenous production from the caecal content used as inoculum [50]. Gas production data were analyzed using a mixed model for repeated measurements, including the sample, the time of measurement, and their interaction as fixed effects, and inoculum (donor rabbit) as a random effect. The model was applied using the PROC MIXED procedure in the SAS package (SAS Inst. Inc., Cary, NC, USA). A heterogeneous compound symmetry structure was fitted as it yielded the lowest value of the Schwarz Bayesian criterion [57]. When the effect of sample or its interaction with the time of measurement was significant, a Dunnett's test was used to compare the value of each macroalgae product with those of the reference ingredients (untreated cereal straw and SBP). Relationships between gas production at 24 h and chemical composition of samples were tested by linear and quadratic correlation analyses using the CORR and GLM procedures in the SAS package. The VFA production at 24 h was analyzed with a mixed model including the sample as a fixed effect and that of inoculum (donor rabbit) as a random effect. Dunnett's test was used to compare the value of each macroalgae product with those of the reference ingredients (straw and SBP).

3. Results and Discussion

3.1. Chemical Composition

The chemical composition of the dehydrated macroalgae varied widely (Table 2). The coefficients of variation ranged from 21% for TDF to 66% for RES (100-ash-CP-TDF). The chemical composition of dehydrated *H. elongata* was found to be rather similar to that of samples previously collected in Galicia, but dehydrated *S. latissima* and *U. pinnatifida* showed different composition (Table 3). As already pointed out, the chemical composition of macroalgae varies depending on geographical location, season, environmental factors, and stressors [21,25,26,58,59]. The chemical composition of both types of extracts was also very variable, with coefficients of variation of 8 for NDF and 106% for SF.

The ash contents of the dehydrated macroalgae were very high (20.8–50.1% DM), which is consistent with the results reported by other authors for these macroalgae species (Table 3), indicating a much higher mineral content than in terrestrial plants [25]. In this respect, it is important to take into account the mineral content of each macroalgae for incorporation in diets, mainly due to the iodine and heavy metals contents [58]. The CP of the dehydrated macroalgae ranged between 7.10 and 16.4% DM, which is consistent with previous reports (5–15% for brown and 10–25% for green algae [23], and with the values previously obtained for these macroalgae in Galicia (Table 3). *Ulva* spp. (16.4%) and *U. pinnatifida* (14.8%) contained almost twice as much protein as SBP (7.99%), and it has been reported that their CP contains relevant proportions of leucine and valine [60,61]. However,

CP content in all macroalgae tested was lower than other protein feeds commonly used in livestock feeding, as sunflower meal or soybean meal.

In the present study, the TDF content varied depending on the species of macroalgae and the type of extract. Dehydrated macroalgae contained large amounts of TDF (28.6–52.7%), although not as high as cereal straw (78.1%) or SBP (71.7%). However, in contrast to straw, macroalgae TDF contained approximately equal parts of NDF and SF (NDF: from 17.1 to 31.3% for macroalgae and 74.3% for straw; SF: from 11.6 to 22.5% for macroalgae and 3.79% for straw), being the values of the macroalgae closer to those of SBP (36.5% NDF and 35.2% of SF). Unlike SBP, some dehydrated macroalgae contained remarkable amounts of ADL (3.63–14.2% for macroalgae; 1.91% for SBP), which may be associated with polyphenolic compounds other than lignin (such as phlorotannins), as lignin is only found in red macroalgae [62]. The results for TDF, although very variable, are broadly consistent with previous reports for samples collected in Galicia (Table 3). The results obtained for SF and other components of TDF are not easy to compare with previously published data because of the variable methods used for determination of macroalgae fibre components in different studies [23,25,26].

Table 2. Chemical composition (g/100 g; dry matter basis) of macroalgae products and reference ingredients (untreated cereal straw and sugar beet pulp) [1].

	Ash	CP	TDF	NDF	SF	ADF	ADL	RES	RES + SF
Dehydrated macroalgae									
Fucus vesiculosus	23.1	10.6	52.7	31.3	21.4	30.7	14.2	13.6	35.0
Himanthalia elongata	41.4	9.51	41.7	19.4	22.3	17.8	13.3	7.39	29.7
Saccharina latissima	20.8	7.10	43.1	22.6	20.5	10.1	3.63	29.0	49.5
Ulva spp.	27.7	16.4	44.1	21.6	22.5	14.7	7.87	11.8	34.3
Undaria pinnatifida	50.1	14.8	28.6	17.1	11.5	11.6	7.10	6.5	18.0
Hydrolyzed macroalgae									
Fucus vesiculosus	28.9	10.6	53.2	30.2	23.0	21.3	15.9	7.3	30.3
Himanthalia elongata	45.5	8.92	40.9	15.3	25.6	16.4	12.5	4.7	30.3
Laminaria ochroleuca	43.4	8.36	33.0	11.2	21.8	11.4	3.85	15.2	37.0
Ulva spp.	39.1	13.0	34.1	5.63	28.5	6.07	4.19	13.8	42.3
Undaria pinnatifida	58.2	14.2	21.3	5.80	15.5	5.68	4.66	6.3	21.8
Aqueous extract									
Fucus vesiculosus	28.7	8.23	10.4	4.83	5.60	4.72	4.32	52.7	58.3
Himanthalia elongata	55.1	3.74	29.9	0.00	29.9	0.14	0.17	11.3	41.2
Saccharina latissima	13.6	3.49	64.7	0.00	64.7	0.00	0.00	18.2	82.9
Undaria pinnatifida	68.7	8.91	18.6	0.26	18.3	0.17	0.16	3.8	22.1
Hydrolyzed extract									
Fucus vesiculosus	32.0	9.22	29.5	1.61	27.9	1.51	1.29	29.3	57.2
Laminaria ochroleuca	44.4	2.37	2.70	0.54	2.16	0.82	0.77	50.5	53.1
Saccharina latissima	19.3	4.09	2.94	0.00	2.94	0.24	0.18	73.7	76.6
Ulva spp.	34.1	4.88	30.2	0.00	30.2	0.09	0.06	30.8	61.0
Mastocarpus stellatus	31.8	10.4	43.4	2.09	41.3	0.14	0.14	14.4	55.7
Reference ingredients									
Untreated cereal straw	8.80	2.90	78.1	74.3	3.79	43.8	4.20	10.2	13.9
Sugar beet pulp	4.70	7.99	71.7	36.5	35.2	23.6	1.91	15.6	50.8

[1] **Crude protein**: [Nitrogen] × 5.0. **TDF**: total dietary fibre. **NDF**: neutral detergent fibre. **SF:** soluble fibre (TDF − NDF). **ADF**: acid detergent fibre. **ADL**: acid detergent lignin. **RES:** 100 − (ash + CP + TDF).

The enzymatic treatment of the macroalgae (hydrolyzed macroalgae products) did not substantially change their chemical composition, especially in *F. vesiculosus* and *H. elongata*. Both the ash (28.9–58.2%) and CP content (8.36–14.2%) were similar to those of the dehydrated samples. In some cases, there was a slight reduction in TDF content due to a lower NDF (5.63–30.2%) but a higher SF level (15.5–28.5%), as it was observed for *Ulva* spp. and *U. pinnatifida*.

Table 3. Review of published data for chemical composition (g/100 g; dry matter basis) of the macroalgae tested in this study.

Location and Reference	Macroalgae	Ash	Crude Protein	Total Dietary Fibre
Galicia, Spain [25]	*Himanthalia elongata*	36.4	14.1	37.1
	Saccharina latissima	34.8	25.7	30.2
	Mastocarpus stellatus	25.0	21.3	31.7
Galicia, Spain [63]	*Himanthalia elongata*	31.0	6.80	39.0
	Laminaria ochroleuca	33.0	8.5	45.0
	Undaria pinnatifida	35.0	20.5	39.0
Galicia, Spain [42]	*Himanthalia elongata*	33.2	7.50	36.0

As expected, both types of extractions influenced the composition of the extract obtained, although in a slightly different way for each macroalgae. No information is available in the literature about the composition of the same extracts for accurate comparisons. The ash content of the extracts ranged from 13.6 to 68.7% DM, and it was numerically higher than that in the corresponding dehydrated macroalgae in most samples. In contrast, the CP content of most extracts was much lower than in the dehydrated macroalgae. For *H. elongata* and *S. latissima*, it was around half in the aqueous extract than in the dehydrated macroalgae (decreased from 9.51% to 3.74%, and from 7.10% to 3.49%, respectively); and the CP reduction was much marked for *Ulva* spp., decreasing from 16.4% in the dehydrated samples to 4.88% in the hydrolyzed extract. In contrast, both aqueous and hydrolyzed extracts of *F. vesiculosus* contained only slightly lower amounts of CP (8.23 and 9.22%, respectively) than the dehydrated macroalgae (10.6%), and CP reductions in the aqueous extract of *U. pinnatifida* were intermediate (8.91 and 14.8% for the extract and the dehydrated macroalgae, respectively). These results suggest a high variability between macroalgae in the CP extraction efficiency, even using the same extraction procedure for all samples. In order to analyze the dietary fibre in the different extracts, it is important to take into account the RES fraction (calculated as RES = 100 − (ash + CP + TDF)), which was very high in some extracts. Considering the low fat and sugar contents usually reported for macroalgae [59,64–66], the RES fraction probably contained mainly soluble carbohydrates, which could not be identified, although in some cases they accounted for a high proportion of the macroalgae. The RES fraction would include the SF that is not precipitated by ethanol or other soluble compounds [67]. In brown macroalgae, such as *S. latissima*, this fraction may correspond to laminarin (1,3-β-D-glucans), which is soluble in water, and alginate (1,4-D-mannuronic acid combined with 1,4-α-L-glucuronic acid), which is soluble at pH between 6 and 9, and/or mannitol [25,68]. These results showed that the standard techniques used to characterize fibre fractions in terrestrial plants do not enable precise quantification of the composition of macroalgae products [25,58,59]. The RES was remarkable in *S. latissima* (29.0% for dehydrated macroalgae and 73.7% for hydrolyzed extract), *F. vesiculosus* (52.7 and 29.3% for aqueous and hydrolyzed extracts, respectively), and the hydrolyzed extracts of *Ulva* spp. (30.8%) and *L. ochroleuca* (50.5%). Therefore, if the RES is considered part of the TDF, and the sum of SF and RES fractions is calculated (Table 2), both the aqueous and hydrolyzed extracts were characterized by high soluble polysaccharides (22.1–82.9%) and very low NDF contents (0–4.83%). These results were expected, as the aim of these extraction processes was to recover the polysaccharides of interest due to their properties and prebiotic potential. Except for *U. pinnatifida*, the sum (RES + SF) was similar or even greater than that of SBP, which is promising due to the fermentation potential of these macroalgae [30,32,69]. Of all the macroalgae considered, *S. latissima* deserves special attention because of the high values of the RES + SF fraction observed in both the aqueous (82.9%) and the hydrolyzed (76.6%) extract, probably due to its high content of laminarin in agreement with the high glucose content in the aqueous extract (80.5%: unpublished data).

3.2. In Vitro Digestibility and Estimated Energy Content

The values of ivIDMd of dehydrated macroalgae ranged from 38.4 to 73.2%, being all higher than that for SBP (43.3%, except in *F. vesiculosus.* Table 4). When the macroalgae were hydrolyzed the ivIDMd increased by 25% on average. In most dehydrated macroalgae, the values of ivFDMd were similar (42.2–75.0%) than those of SBP (76.8%). The exceptions were *F. vesiculosus* and *H. elongata*, both of which had relatively high sulphate contents (4–7%: unpublished data), and the latter also had a high fucose content (31.5%: unpublished data) probably associated with fucoidans. However, the ivFDMd values were higher in all macroalgae than in the cereal straw (19.8%). Conversely, CP digestibility (ivFCPd) was lower in all macroalgae than in SBP (22.6–59.0% for dehydrated macroalgae and 68.1% for SBP), with some macroalgae (i.e., *F. vesiculosus* and *H. elongata*) having values close to that of cereal straw (17.7%). Both DM and CP digestibility increased when the macroalgae were hydrolyzed (by 20% ivFDMd, and 80% ivFCPd, on average, except in *F. vesiculosus*). The low *in vitro* CP digestibility is consistent with the low *in vivo* CP digestibility reported for *S. latissima* and *Ulva* spp. in rats [70], despite these samples showing the numerically highest values *in vitro* in our study. The ivFDMd and ivFCPd values of the dehydrated macroalgae were negatively correlated with ADF content (r = −0.97; p = 0.005; n = 5) and the protein associated with TDF (r = −0.96; p = 0.011; n = 5), respectively, but they were not correlated with either SF or the sum of RES + SF fractions ($p \geq 0.35$). The negative correlation between ivFDMd and ADF was previously observed for compound feeds for rabbits based on terrestrial plant ingredients [47].

Table 4. *In vitro* ileal (ivIDMd) and faecal digestibility of DM (ivFDMd), *in vitro* facecal protein digestibility (ivFCPd), and estimated nutritional value of macroalgae products [1].

	In Vitro Digestibility (%)			Estimated Nutritive Value		
	ivIDMd	ivFDMd	ivFCPd	DMd (%)	GEd (%)	DE (MJ/kg Dry Matter)
Dehydrated macroalgae						
Fucus vesiculosus	38.4	42.2	22.6	39.5	39.8	7.96
Himanthalia elongata	57.6	63.3	25.1	60.1	59.8	11.1
Saccharina latissima	66.0	74.3	55.6	70.9	70.3	12.8
Ulva spp.	65.2	75.0	59.0	71.9	71.2	12.9
Undaria pinnatifida	73.2	74.6	49.8	71.2	70.6	12.8
Hydrolyzed macroalgae						
Fucus vesiculosus	43.2	42.7	14.1	39.9	40.3	8.0
Himanthalia elongata	73.0	73.3	43.2	69.9	69.3	12.6
Laminaria ochroleuca	73.5	74.0	64.8	70.6	70.0	12.7
Ulva spp.	86.9	91.5	100	87.8	86.6	15.3
Undaria pinnatifida	93.0	92.1	100	88.4	87.2	15.4
Aqueous extract						
Fucus vesiculosus	99.6	98.8	100	94.9	93.6	16.4
Himanthalia elongata	99.7	99.4	100	95.5	94.1	16.5
Saccharina latissima	98.0	99.6	100	95.7	94.3	16.6
Undaria pinnatifida	99.7	99.7	100	95.8	94.4	16.6
Hydrolyzed extract						
Fucus vesiculosus	93.5	97.9	100	94.0	92.7	16.3
Laminaria ochroleuca	99.5	98.7	100	94.8	93.5	16.4
Saccharina latissima	99.7	99.2	100	95.3	93.9	16.5
Ulva spp.	99.8	99.0	100	95.1	93.8	16.5
Mastocarpus stellatus	78.6	99.1	100	95.2	93.8	16.5
Reference ingredients						
Untreated cereal straw	19.7	19.8	17.7	17.5	18.5	4.60
Sugar beet pulp	43.3	76.8	68.1	73.4	72.7	13.1

[1] **DMd**: apparent dry matter faecal digestibility (%). **GEd**: apparent gross energy faecal digestibility. **DE**: digestible energy. All values were estimated from equations of Villamide et al. [47].

As expected, the different extracts obtained from the macroalgae were almost completely digested *in vitro* (78.6–99.8% ivIDMd; 97.9–99.7% ivFDMd; 100.0% ivFCPd) due to their soluble nature. However, the polysaccharides quantified in both the TDF and the RES fractions (possibly laminarin, alginate, fucoidans, ulvans) cannot be hydrolyzed by the endogenous enzymes and can only be fermented by the intestinal microbiota. The *in vivo* protein digestibility of these extracts might be higher than in the dehydrated macroalgae although it would not be expected to be high. Specific protein extraction in *Ulva* spp. has been shown to increase *in vitro* proteolysis [71], which was in agreement with the increased ivFCPd observed in our study for the hydrolyzed *Ulva* spp. and *U. pinnatifida* compared with the dehydrated macroalgae, but these results should be confirmed *in vivo.* None of the *in vitro* digestibility values measured in the extracts were correlated with any fibrous fractions analyzed ($p \geq 0.30$; $n = 9$). When considering all of the 19 macroalgae products together, the ivFDMd and ivFCPd values were negatively correlated with NDF (r = -0.96; $p < 0.001$; $n = 19$), and with the protein linked to NDF (r = -0.82; $p < 0.001$; $n = 19$) content, respectively.

The dehydrated macroalgae showed similar values of digestible energy (7.96–12.9 MJ/kg DM) than SBP (13.1 MJ/kg DM), with the exception of *F. vesiculosus*, but higher values than straw (4.60 MJ/kg DM) (Table 4). Hydrolyzation of the macroalgae slightly improved their energy content, except for *F. vesiculosus.* In both types of extracts, DE values were higher (16.5 MJ/kg DM, on average) than that of SBP, which is in good accordance with the high DM digestibility of the extracts, although the real value will depend on their fermentability.

3.3. In Vitro Gas and VFA Production

Values for the macroalgae products and reference materials are shown in Table 5, whereas those for the insoluble residue of the ivIDMd are shown in Table 6.

Table 5. T Gas production after 12, 24, and 48 h of *in vitro* incubation of macroalgae products and reference ingredients using caecal rabbit digesta as inoculum ($n = 4$) [1].

Sample	Gas Production (mL/g Dry Matter)		
	12 h	24 h	48 h
Dehydrated macroalgae			
Fucus vesiculosus	6.40 [a]	8.25 [a]	8.25 [a]
Himanthalia elongata	5.98 [a]	10.3 [a]	25.6 [a]
Saccharina latissima	7.23 [a]	55.8 [c]	95.6 [c]
Ulva spp.	12.7 [b]	16.6 [a]	20.7 [a]
Undaria pinnatifida	3.70 [a]	3.75 [a]	19.4 [a]
Hydrolyzed macroalgae			
Fucus vesiculosus	2.58 [a]	2.58 [a]	6.43 [a]
Himanthalia elongata	2.63 [a]	2.63 [a]	14.3 [a]
Laminaria ochroleuca	12.9 [b]	23.3 [a]	30.3 [a]
Ulva spp.	11.4 [a b]	11.4 [a]	17.9 [a]
Undaria pinnatifida	3.18 [a]	3.18 [a]	11.0 [a]
Aqueous extract			
Fucus vesiculosus	6.78 [a]	8.20 [a]	20.7 [a]
Himanthalia elongata	5.68 [a]	22.2 [a]	45.0 [b]
Saccharina latissima	8.00 [ab]	90.2 [c]	145 [d]
Undaria pinnatifida	6.15 [a]	6.15 [a]	22.8 [a]
Hydrolyzed extract			
Fucus vesiculosus	10.0 [ab]	29.6 [a]	49.6 [b]
Laminaria ochroleuca	7.15 [a]	73.5 [c]	103 [d]
Saccharina latissima	10.7 [ab]	112 [d]	167 [e]
Ulva spp.	8.60 [a]	30.0 [b]	46.3 [b]
Mastocarpus stellatus	5.70 [a]	11.4 [a]	23.9 [a]
Reference ingredients			
Untreated cereal straw	5.08 [a]	5.08 [a]	8.35 [a]
Sugar beet pulp	15.0 [b]	67.7 [c]	125 [d]

[1] The effects of sample, measurement time (hours), and their interaction were all significant ($p < 0.001$). Pooled standard errors of means at 12, 24, and 48 h were 1.53, 5.35, and 5.46, respectively. For each measurement time, different superscripts lowercase letters (within each column) indicate whether each mean is different ($p < 0.05$) from the reference ingredients (untreated cereal straw and sugar beet pulp).

Table 6. Gas production after 12, 24, and 48 h of *in vitro* incubation of the insoluble residues of the 2-step *in vitro* digestibility of macroalgae (dehydrated and enzymatically hydrolyzed) and of reference ingredients using caecal rabbit digesta as inoculum (*n* = 4) [1].

Sample	Gas Production (mL/g Dry Matter)		
	12 h	24 h	48 h
Insoluble residue of dehydrated algae			
Fucus vesiculosus	5.05 [a]	5.15 [a]	6.20 [a]
Himanthalia elongata	4.98 [a]	5.43 [a]	18.3 [a]
Saccharina latissima	4.10 [a]	5.60 [a]	34.6 [b]
Ulva spp.	1.68 [a]	1.63 [a]	12.2 [a]
Undaria pinnatifida	3.85 [a]	4.63 [a]	10.3 [a]
Insoluble residue of hydrolyzed algae			
Fucus vesiculosus	3.00 [a]	3.00 [a]	3.83 [a]
Himanthalia elongata	3.65 [a]	3.65 [a]	10.5 [a]
Laminaria ochroleuca	3.23 [a]	3.23 [a]	4.33 [a]
Ulva spp.	4.15 [a]	4.95 [a]	7.80 [a]
Undaria pinnatifida	4.20 [a]	4.20 [a]	4.80 [a]
Insoluble residue of the reference ingredients			
Untreated cereal straw	2.90 [a]	2.90 [a]	2.90 [a]
Sugar beet pulp	3.70 [a]	7.90 [a]	38.6 [b]
Reference ingredients			
Untreated cereal straw	5.08 [a]	5.08 [a]	8.35 [a]
Sugar beet pulp	15.0 [b]	67.7 [b]	125 [c]

[1] The effects of sample, the measurement time (h) and their interaction were all significant (*p* < 0.001). Pooled standard errors of means at 12, 24, and 48 h: 1.53, 5.35, and 5.46, respectively. For each measurement time, different superscripts lowercase letters (within each column) indicate whether each mean is different (*p* < 0.05) from the reference ingredients (untreated cereal straw and sugar beet pulp). The fermentability of the insoluble residue of the 2-step *in vitro* digestibility were assessed only for samples with an *in vitro* dry matter ileal digestibility lower than 93%.

The different kinetics of gas production observed in the samples precluded the data being fitted with a single mathematical model, and comparison between samples was thus impossible. Accordingly, only data for 12, 24, and 48 h of incubation are shown for comparison of the samples, as they were more closely correlated with the real *in vivo* fermentation and fibre digestibility in the rabbit (unpublished results). At 48 h, 14 out of 33 samples (macroalgae products + reference ingredients + insoluble residues of 2-step *in vitro* digestibility) have reached the whole potential gas production. When data were analyzed as repeated measures, *in vitro* gas production was influenced by the incubated sample, measurement time and their interaction (*p* < 0.001).

Surprisingly, the two samples that showed the closest (*p* > 0.05) gas production than SBP after 12 h were the dehydrated *Ulva* spp. and the hydrolyzed *L. ochroleuca* samples (Table 5), whereas for the hydrolyzed *Ulva* spp., both extracts of *S. latissima* and the hydrolyzed extract of *F. vesiculosus* showed intermediate values between SPB and straw. At 24 h, gas production in dehydrated *S. latissima*, the aqueous extract of *S. latissima* and the hydrolyzed extract of *L. ochroleuca* was similar to that in SBP, while gas production in the hydrolyzed extract of *S. latissima* was higher than in SBP (*p* < 0.05). Similar results were obtained at 48 h. The results indicated a high fermentability of the different *S. latissima* products, which is probably related to the high content in laminarin and alginate previously reported [72], especially when these polysaccharides are concentrated, as it may occur in the extracts. Laminarin is a non-sulphated water-soluble storage polysaccharide composed of 1,3-β-D-glucans with β1,6 ramifications, and alginate is composed of 1,4-β-D-manuronic acid and 1,4-β-L-guluronic acid and is water soluble at pH 6–9; however, both cannot be hydrolyzed by endogenous enzymes and can be only used by intestinal microbiota [73]. *S. latissima* was also previously found to yield a relatively high level of ruminal gas production *in vitro*, lower than SBP but higher than alfalfa meal [59]. By contrast, gas production was lower in the macroalgae containing relatively high levels of sulphated polysaccharides

(ulvans and fucoidans), such as *F. vesiculosus* and *Ulva* spp., which had been also reported to be less fermentable in other studies [74,75].

The other macroalgae products yielded similar gas production to that produced by straw, suggesting a low fermentability, with the extracts of *H. elongata* (aqueous), *F. vesiculosus* (hydrolyzed), and *Ulva* spp. (hydrolyzed) yielding intermediate values between SBP and straw at 48 h.

The different fermentability of samples at 12 h suggests the need of adaptation for the microbiota, especially to the most purified extracts, in which the chemical composition is more homogeneous and different from that of substrates usually fermented by the rabbit intestinal microbiota. The caecal inocula were obtained from rabbits that were not fed macroalgae, and the adaptation required would have delayed the beginning of the fermentation process. The need for substrate adaptation by the rabbit intestinal microbiota has been already observed when using a very specific substrate (cellobiose) [50].

Most of the insoluble residues of ivIDMd yielded similar gas production to the cereal straw (Table 6), independently of the fermentation time, which is consistent with the higher fermentability of soluble compared with insoluble fibre in rabbits [76]. Only the insoluble residues of *S. latissima* and SBP produced more gas than the cereal straw not subjected to *in vitro* digestion but less than SBP.

Although a positive correlation between the gas production at 24 h and the RES fraction was observed for the dehydrated macroalgae (r = 0.96; p = 0.011; n = 5), no correlation was found with SF or the ivIDMd, ivFDMd, and ivFCPd values ($p \geq 0.54$). The gas production at 24 h of the 9 extracts (aqueous and hydrolyzed) was positively correlated with the sum of RES + SF (r = 0.72; p = 0.028; n = 9), but was not correlated with SF or ivIDMd, ivFDMd, and ivFCPd ($p \geq 0.34$). When the relationships between the gas production at 24 h of the 19 macroalgae products and their chemical composition were analyzed, positive correlations were again observed with the RES fraction (r = 0.71; p < 0.001; n = 19) and with the sum (RES + SF), both linearly (R^2 = 0.62; p < 0.001; n = 19) and quadratically (R^2 = 0.68; p = 0.095. Figure 1).

Gas production at 24 h was also negatively correlated with CP (r = -0.69; p = 0.001; n = 19) and ADL content (r = -0.51; p = 0.025; n = 19), but less significant correlations were observed with ivIDMd, ivFDMd, and ivFCPd (r = 0.37–0.40; p = 0.086–0.12; n = 19), and no correlations were observed with SF fraction (p = 0.85). These results indicate the complexity of characterizing macroalgae products with the standard techniques used for terrestrial plant ingredients. In addition, solubility does not equate to fermentability in these samples, which could be due to the presence of either substances that inhibit enzyme action [66,77], or highly fermentable compounds not collected in the TDF fraction. The negative influence of ADL on *in vitro* gas production is similar to that observed by Bikker et al. [59] for macroalgae and to that observed in terrestrial plant ingredients [78], although the chemical meaning of ADL in macroalgae may not be the same than in terrestrial plants.

The *in vitro* VFA production was determined only in the most fermentable macroalgae products and the two reference materials (Table 7). As expected, gas and total VFA production at 24 h were positively correlated (r = 0.84; p = 0.018; n = 9). All products derived from *S. latissima* showed total VFA production similar to that for SBP. Both extracts of *S. latissima* had increased VFA production compared with the dehydrated macroalgae, probably due to the higher concentration of fermentable polysaccharides such as laminarin, as previously observed *in vitro* in ruminants [79,80]. The other analyzed macroalgae products had lower (p < 0.05) total VFA production than *S. latissima* products.
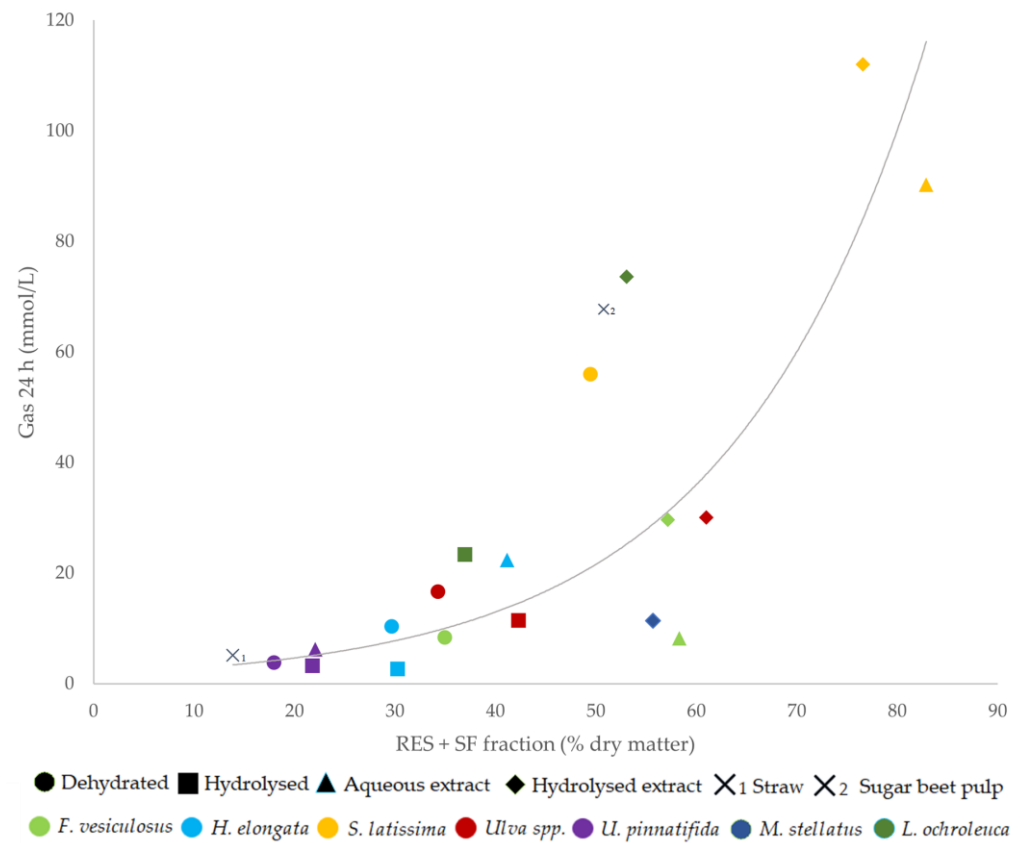
Figure 1. Relationship between the *in vitro* gas production at 24 h of incubation with caecal content from rabbits (mmol/L) and the sum of RES + SF fraction (% dry matter) in the 19 macroalgae products and two reference ingredients (sugar beet pulp and straw). $R^2 = 0.68$; linear effect: $p < 0.001$, quadratic effect: $p = 0.095$; residual standard deviation = 20.1; $n = 19$. RES = 100-ash-CP-TDF.

Table 7. *In vitro* total volatile fatty acid production (VFA; mmol/L) and molar proportions of individual VFA after incubating macroalgae products and reference ingredients with caecal inoculum from rabbits for 24 h ($n = 4$) [1].

	Total VFA	Molar Proportion (mol/100 mol)						
		Ac	Pr	But	Ibut	Val	Ival	Cap
Dehydrated macroalgae								
Saccharina latissima	37.9 [b]	70.1 [b]	25.0	4.67 [a]	0.08 [a]	0.12	0.03	0.00
Aqueous extract								
Himanthalia elongata	14.8 [a]	62.1 [a]	7.40	27.1 [b]	2.90 [a]	0.47	0.00	0.00
Saccharina latissima	42.6 [b]	67.8 [b]	20.7	7.60 [a]	1.22 [a]	1.07	0.95	0.60
Hydrolyzed extract								
Fucus vesiculosus	21.1 [a]	82.5 [b]	10.2	4.07 [a]	1.62 [a]	0.57	1.07	0.00
Laminaria ochroleuca	18.0 [a]	71.2 [b]	13.8	14.0 [b]	0.67 [a]	0.35	0.00	0.00
Saccharina latissima	52.2 [b]	74.7 [b]	21.2	3.77 [a]	0.15 [a]	0.12	0.03	0.00
Ulva spp.	20.5 [a]	66.2 [b]	14.6	8.00 [a]	10.7 [b]	0.37	0.05	0.00
Reference ingredients								
Untreated cereal straw	4.20 [a]	84.0 [b]	10.2	5.85 [a]	0.00 [a]	0.00	0.00	0.00
Sugar beet pulp	47.4 [b]	83.4 [b]	14.1	2.20 [a]	0.17 [a]	0.08	0.00	0.00
SEM (n = 4)	4.73	6.51	5.57	2.94	0.75	0.33	0.37	0.20

[1] **Ac**: acetate. **Pr**: propionate. **But**: butyrate. **Ibut**: isobutyrate. **Val**: valerate. **Ival**: isovalerate. **Cap**: caproate. Within each variable different superscripts lowercase letters (within each column) indicate whether each mean is different ($p < 0.05$) from the reference ingredients.

Saccharina latissima products also produced high proportions of propionate (25.0, 20.7, and 21.2% for the dehydrated sample, aqueous extract, and hydrolyzed extract, respectively), but they did not differ with that produced by SBP (14.1%) or straw (10.2%). The fermentation of the aqueous extract of *H. elongata* and the hydrolyzed extract of *L. ochroleuca* resulted in higher ($p < 0.05$) proportions of butyrate than the reference ingredients, which may be associated with the fucoidan content [81]. A high proportion of butyrate is of interest due to its benefits on gut health, as it is known to be the main energy source for colonocytes [82]. In rabbits, the ileal concentration of butyrate has been associated with better growth traits when cellobiose was supplied in the diet [83]. The hydrolyzed extract of *Ulva* spp. produced a remarkable amount of isobutyrate (10.7%), much higher than SBP (0.17%) or any other macroalgae product.

Macroalgae polysaccharides, particularly laminarin and fucoidan, have been shown to have a prebiotic effect in several *in vitro* and *in vivo* studies (for a review, [32]), but their effects on VFA proportions is variable. In most studies, laminarin (derived from macroalgae such as *S. latissima* and *L. ochroleuca*) has been found to yield high levels of gas production and increased levels of acetate [84,85], propionate [84–86], and butyrate [86,87]. Fermentation of fucoidan, present in macroalgae such as *F. vesiculosus* and *H. elongata*, among others, also showed an increase in the proportions of acetate and butyrate [88], but a decrease in that of propionate [86,88]. It is important to consider that the *in vitro* VFA proportions are influenced by the incubated macroalgae, but probably also by the source of the inoculum used [49].

3.4. Minimal Inhibitory Concentrations

Most of the macroalgae products tested did not inhibit the bacterial growth at the maximal concentration tested (8.2 mg/mL. Table 8).

Table 8. Minimal inhibitory concentrations (MIC; mg/mL) of the macroalgae products tested against aerobic and anaerobic bacteria [1].

	EC434	EC727	S. ty	S. ent	EC R1	EC R2	LM	Pse	C. per	C. dif
Dehydrated macroalgae										
Fucus vesiculosus	-	-	-	-	-	-	-	-	-	-
Himanthalia elongata	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	-	-
Saccharina latissima	-	-	-	-	-	-	-	-	-	-
Ulva spp.	-	-	-	-	-	-	-	-	-	-
Undaria pinnatifida	8.2	8.2	8.2	8.2	8.2	8.2	2.05	8.2	-	-
Hydrolyzed macroalgae										
Fucus vesiculosus	-	-	-	-	-	-	-	-	-	-
Himanthalia elongata	-	-	-	-	-	-	-	-	-	-
Laminaria ochroleuca	-	-	8.2	8.2	-	8.2	8.2	8.2	-	-
Ulva spp.	-	-	-	-	-	-	8.2	-	-	-
Undaria pinnatifida	-	-	-	-	-	-	-	-	-	-
Aqueous extract										
Fucus vesiculosus	-	-	-	-	-	-	-	-	-	-
Himanthalia elongata	-	-	-	-	-	-	-	-	-	-
Saccharina latissima	-	-	-	-	-	-	-	-	-	-
Undaria pinnatifida	-	-	-	-	-	-	-	-	-	-
Hydrolyzed extract										
Fucus vesiculosus	-	-	-	-	-	-	-	-	-	-
Laminaria ochroleuca	-	-	-	-	-	-	-	-	-	-
Saccharina latissima	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	-	-
Ulva spp.	-	-	-	-	-	-	-	-	-	-
Mastocarpus stellatus	-	-	-	-	-	-	-	-	-	-

[1] **EC 434**: *Escherichia coli* CECT 434. **EC727:** *Escherichia coli* CECT 727. **S. ty:** *Salmonella typhimurium.* **S. ent:** *Salmonella enteritidis.* **EC R1:** *Escherichia coli* R1 (LREC). **EC R2:** *Escherichia coli* R2 (LREC). **LM:** *Listeria monocytogenes.* **Pse:** *Pseudomonas aeruginosa.* **C. per:** *Clostridium perfringens.* **C. dif:** *Clostridium difficile.*

Two of the dehydrated macroalgae (*H. elongata* and *U. pinnatifida*) inhibited bacterial growth at the maximal concentration (8.2 mg/mL, except 2.05 mg/mL for *U. pinnatifida* against LM), but their products did not have the same effect. Previous findings indicate that these macroalgae could have some antimicrobial properties [89,90]. Although it is not known what causes the antimicrobial effect of some macroalgae, it has been mainly attributed to the phenolic compounds [90,91]. In the present study, the extracts were obtained with the objective of increasing digestibility and/or fermentability and concentrating the SF of the macroalgae, so any antimicrobial effect may have disappeared at least partly in these extracts because of the absence of these compounds.

Within the macroalgae products, hydrolyzed *L. ochroleuca* and the hydrolyzed extract of *S. latissima* also showed inhibitory responses when tested at the maximal concentrations. Unfortunately, a sample of dehydrated *L. ochroleuca* was not available for testing, and it is possible that it could also show an inhibitory effect. By contrast, dehydrated *S. latissima* did not show any inhibitory effect, suggesting that the positive effect observed in the hydrolyzed extract may be associated with other compounds concentrated in this macroalgae product [90]. Further studies with different solvents and extracts are required to enable solid conclusions to be reached regarding the potential inhibitory effects of these macroalgae. Nonetheless, the positive results appeared only at the maximal concentration, which would correspond in vivo to an inclusion of the macroalgae at a proportion of 2.5% in the diet. Moreover, it must be taking into account that *H. elongata*, *U. pinnatifida*, *L. ochroleuca*, and *S. latissima* are brown macroalgae with a high iodine content, which limits the amount that could be included in rabbit feed (Regulation EC 1334/2003).

4. Conclusions

Overall, the dehydrated macroalgae tested in this study were notable for their high content of minerals, and especially as an interesting source of dietary fibre, particularly of soluble fibre. The in vitro digestibility and caecal fermentation of dehydrated *S. latissima* were similar to those obtained for sugar beet pulp. All *S. latissima* products showed high fermentation potential and VFA production, especially both types of extracts. The *H. elongata* aqueous extract was remarkable due to its high in vitro butyrate production that can be of great interest for improving gut health. The MIC results did not indicate any clear inhibition of the pathogens tested by the macroalgae products. Based on our findings, the macroalgae tested appear to have a potentially prebiotic effect, rather than a direct antimicrobial activity. However, these results must be confirmed in vivo, in order to observe the real benefits of feeding macroalgae during the rabbit weaning period.

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