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In vitro ruminal fermentation and methane production of different seaweed species

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Highlights

The polyphenols content and *in vitro* rumen fermentation of seven seaweed species was investigated

Seaweed collected in autumn had higher polyphenols and lower degradability than those from spring

Palmaria palmata had the highest rumen degradability and *Pelvetia canaliculata* the lowest

Diversity and abundance of ruminal microbiota promoted by the three red seaweeds were comparable

ABSTRACT

Seaweeds have potentials as alternative feed for ruminants, but there is a limited knowledge on their nutritive value. Seven seaweed species collected along the coast above the Arctic circle of Norway, both in spring and autumn, were assessed for nutrients and total polyphenols (TEP) content, gas production kinetics and *in vitro* rumen fermentation in batch cultures of ruminal microorganisms. The seaweeds were three red species (*Mastocarpus stellatus, Palmaria palmata* and *Porphyra sp.*), three brown species (*Alaria esculenta, Laminaria digitata* and *Pelvetia canaliculata*) and one green species (*Acrosiphonia sp.*). Additionally, the abundance and diversity of total bacteria, protozoa and archaea in the cultures with the three red seaweeds collected in spring were analyzed by quantitative PCR and PCR-DGGE, respectively. The crude protein (CP) content varied widely. Pelvetia had the greatest ($P < 0.001$) ether extract (EE) content. Non-structural carbohydrates (NSC) content varied from 135 to 541 g/kg DM with brown seaweeds having the greatest values. Ash and CP contents were higher in spring than in autumn ($P = 0.020$ and 0.003, respectively), whereas concentrations of EE and NSC were not affected by collecting season ($P = 0.208$ to 0.341). The TEP

values ranged from 1.46 to 50.3 mg / g dry matter (DM), and differed ($P < 0.001$) among seaweed species and collecting season, being greater in autumn than in spring. The DM effective degradability (DMED), estimated from gas production parameters for a rumen passage rate of 3.0 % per h, ranged from 424 to 652 g / kg, the highest values were recorded for *Mastocarpus stellatus and Porphyra sp.* The lowest DMED values were registered for *Pelvetia canaliculata* and *Acrosiphonia* sp. In 24-h incubations (500 mg DM), *Palmaria palmata* had the highest ($P < 0.05$) volatile fatty acids (VFA) and methane production (4.34 and 0.761 mmol, respectively) and the lowest ($P < 0.05$) final pH values and acetate to propionate ratios (6.57 and 2.34, respectively). There were no differences ($P > 0.05$) among the other seaweeds in VFA production, but *Porphyra sp.* had the second highest methane production ($P < 0.05$; 0.491 mmol) compared with the other seaweeds (0.361 mmol; averaged value). The methane / total VFA ratio was not affected ($P > 0.05$) by either seaweed species or the collection season. Higher final pH ($P < 0.05$) and lower ($P < 0.05$) methane and VFA production, ammonia-N concentrations and DMED values were promoted by the fermentation of seaweed collected in autumn compared with those from spring. Among the red seaweeds, there were no species-specific differences ($P > 0.05$) in the abundance or the diversity of total bacteria, protozoa and archaea. In the PCR-DGGE analysis, samples were separated by the incubation run for all microbial populations analyzed, but not by seaweed species. The results indicate that seaweed species differ markedly in their *in vitro* rumen degradation, and that samples collected in autumn had lower rumen degradability than those collected in spring.

Abbreviations: AGPR, average gas production rate; CP: crude protein; DM, dry matter; DMED, DM effective degradability; $DMD_{72,}$ DM degradability after 72 h of incubation; EE, ether extract; NDF, neutral detergent fibre; NSC, non-structural carbohydrates; PCR, polymerase chain reaction; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; qPCR, quantitative PCR; TEP: total extractable polyphenols; $TDMD_{72}$, True DMD_{72}; VFA, volatile fatty acids.

Key words: seaweeds; chemical composition; batch cultures; rumen fermentation; microorganisms; methane

1. Introduction

The world human population is estimated to reach 9.1 billion by 2050, and the demand for meat and dairy products is rapidly increasing (Steinfeld and Wassenaar, 2007). In this context, the search for alternative feed sources for livestock, such as unconventional feedstuffs like seaweeds is a priority (Odegard and van der Voet, 2014). About 71% area of earth's surface is covered by ocean, which holds about 96.5% of the planet's hydrosphere and a large fraction of body of water and its biota remains unexploited. Seaweeds are primary producers growing along coastal habitats under natural conditions of light and temperature. Cultivation of seaweed do not compete with agricultural land that can be utilized for the production of other valuable crops, do not require freshwater, and aquatic photosynthesis can help buffer the effect of increasing atmospheric CO_2. Moreover, there is an increasing interest in using marine resources like seaweed in animal feeding as sources of protein to replace soya bean meal.

Seaweeds are also interesting for their potential beneficial effects on animal and consumers health (Makkar et al., 2016). However, the use of seaweeds in feeding ruminants is limited due to the lack of information regarding species-specific nutritive value and variability, but also to the presence of substances which could be a challenge to the digestive system of terrestrial animals (Evans and Critchley, 2014; Makkar et al., 2016).

Seaweeds are macroscopic, multicellular marine algae and belong to three distinct evolutionary groups: green (phylum Chlorophyta), brown (phylum Ochrophyta), and red (phylum Rhodophyta). These three phyla differ considerably in their morphology (functional form), physiology, and biochemistry, and therefore also in chemical composition. Chemical composition of seaweed depends on various factors like species, harvesting season, habitat and light and water temperature, among others, and therefore a great variability is reported in the literature (Makkar et al., 2016). Some seaweeds are rich in protein (Tayyab et al., 2016) and others contain secondary compounds with variable biological activities which are almost unknown (Makkar et al., 2016). As livestock production relies heavily on the supply of high-quality protein feeds, with soybean being the most widely used plant protein source in Europe, protein rich seaweeds could potentially be an alternative to soybean protein (Tayyab et al., 2016).

Methane emission from enteric fermentation in animals is of concern worldwide due to its contribution to the accumulation of greenhouse gases in the atmosphere and an energy loss for the host animal (Hook et al., 2010). Recent studies suggest that red and brown seaweed species may have bioactive components with antimethanogenic properties (Belanche et al., 2016a; Kinley et al., 2016), although others (Belanche et al., 2016b) have failed to detect any influence on methane production. Differences in the seaweed species and inclusion rate can explain the variable results. Given the

importance of this topic and the low number of studies in which the influence of seaweeds on methane emissions by ruminants has been assessed, further research is necessary.

The objective of the present study was therefore to provide information on nutrients and phenol composition and assess gas production kinetics, *in vitro* fermentation and methane emission of seven seaweed species (*Alaria esculenta, Laminaria digitate, Pelvetia canaliculata, Mastocarpus stellatus, Palmaria palmate, Porphyra sp.* and *Acrosiphonia sp.*) collected in northern Norway. These seaweed species were chosen primarily based on biomass availability, cultivation potential, biochemical composition (Holdt and Kraan, 2011), but also based on traditional use of seaweed for feeding animals, and observed feeding preference of free-range ruminants along coastal areas grazing on beach cast seaweeds (Bay-Larsen et al. 2016). Huge biomass of *Alaria esculenta* is seasonally available, while biomass of *Palmaria palmata*, *Mastocarpus stellatus* and *Pelvetia canaliculata* are available year-round with different seasonal peaks (Roleda M. Y. personal observation). *Pelvetia canaliculata* has been traditionally used to feed animals during lean season (i.e. winter months), and *Palmaria palmata* has been observed to be preferred by free-range sheep browsing through piles of beach-cast seaweeds (Bay-Larsen et al., 2016). The protein value of these seven species has been recently analysed in dairy cows by Tayyab et al. (2016), and the results showed that *Acrosiphonia sp., Alaria esculenta, Laminaria digitata*, *Mastocarpus stellatus* and *Palmaria palmata* can supply the rumen with high amounts of rumen degradable protein, while *Porphyra sp*. can be used as a source of digestible bypass protein. Seaweeds were collected in two seasons (spring and autumn) to assess seasonal variability of the seaweeds nutrients. In addition, the ruminal microbiota promoted by the fermentation of some of the seaweeds was analyzed.

2. Materials and Methods

2.1. Animals

Two rumen-cannulated Segureña ewes (50.9 ± 2.91 kg body weight; 2 years old) were used as rumen contents' donors for the *in vitro* incubations. Animals were cared and handled by trained personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes). All the experimental procedures were approved by the Animal Welfare Committee at the Estación Experimental del Zaidin (CSIC, Spain; Approval number: 24/05/2016/091). Animals were fed a standard diet composed of alfalfa hay and a commercial concentrate in a 1:1 ratio at 50 g per Kg body weight$^{0.75}$, which corresponded to energy maintenance level (Aguilera et al., 1986). Chemical composition of the diets is shown in Table 1. Experimental diets were offered in two meals at 9:00 h and 18:00 h. Clean water and mineral supplement were always available. Concentrate (Pacsa Sanders, Seville, Spain) was provided as pellets and consisted (g / kg fresh matter) of wheat flour (350), sunflower meal (200), malt sprouts (80), canola meal (130), soybean hulls (200), calcium carbonate (10), rumen-inert fat (25) and sodium chloride (5). The composition (g / kg DM) of the diet was: organic matter (OM) 883, crude protein (CP) 185, neutral detergent fiber (aNDFom) 355, acid detergent fiber (aADFom) 224, ether extract (EE) 43 and 9.25 MJ / kg DM of ME.

2.2. Seaweeds collection and preparation

Seaweeds were collected by hand picking in spring (March) and autumn (October-November) 2014 in Bodø, Norway *(67°19'00'' N, 14°28'60''* E) during low tide. The collected seaweeds were three red species (*Mastocarpus stellatus, Palmaria palmata* and *Porphyra sp.*), three brown species (*Alaria esculenta, Laminaria digitata* and

Pelvetia canaliculata) and one green species (*Acrosiphonia sp.*). The seaweed samples were first cleaned of sand and associated organisms in baths of seawater. Thereafter, the samples were briefly washed in water of decreasing salinity (30%), and finally in freshwater to reduce surface salts. The excess water was drained manually and samples were frozen at -20°C. Finally, the samples were lyophilized and milled through a 1 mm screen with a cutter mill as described by Tayyab et al. (2016).

2.3. In vitro *incubations*

Samples of the lyophilized seaweeds were used as substrates for *in vitro* incubations using batch cultures of rumen microorganisms. Rumen contents were collected from the two sheep before the morning feeding, pooled and immediately taken to the laboratory into thermal flasks. Rumen contents were strained through four layers of cheesecloth and mixed with a buffer solution (Goering and Van Soest, 1970) in a 1:4 ratio (vol / vol) at 39°C under continuous flushing with CO_2. The time required from rumen content collection to inoculation of bottles was < 30 min.

A total of six *in vitro* incubation runs were carried out, three for the samples collected in spring and three for the autumn-collected samples. Four bottles per sample and four bottles without substrate (blanks) were used in each run. Blanks were used to correct the gas production values for gas release from endogenous substrates. Samples (0.500 g) of seaweeds were carefully weighed into 120 ml bottles, and 60 ml of the buffered rumen fluid were anaerobically added into each bottle. Bottles were sealed with butyl rubber stoppers and aluminum caps and incubated at 39º C in a water bath. In two of the four bottles for each seaweed sample and two blanks, pressure and gas volume were measured at 2, 4, 6, 8, 12, 24, 48 and 72 h of incubation using a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ, USA) and a glass-calibrated syringe (Ruthe®, Normax, Marinha Grande, Portugal), respectively. After 72

h, the content of bottles was freeze-dried and the DM residue determined to calculate the DM degradability (DMD_{72}). Finally, the residue was analyzed for $aNDF_{OM}$ to calculate true DM degradability ($TDMD_{72}$; Van Soest et al., 1966).

In the remaining two bottles for each sample, the gas produced after 24 h of incubation was measured as described above and a gas sample (about 5 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of methane. Bottles were then uncapped, the pH was measured immediately (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona. Spain), the fermentation was stopped by placing the bottles in iced-water, and the following samples were taken: 2 ml was added to 2 ml of deproteinising solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per litre) for VFA determination, 1 ml was mixed with 1 ml 0.5 *M* HCl for NH_3-N analysis, and 5 ml were transferred to sterile containers and frozen at -80ºC for further analyses.

2.4. DNA isolation and quantitative PCR and PCR-DGGE analyses

The DNA isolation and molecular analyses were performed on the content of the cultures in which samples of the red seaweeds (*Mastocarpus stellatus, Palmaria palmata and Porphyra sp*.) collected in spring were fermented. These seaweeds were selected for these analyses due to the relatively high CP content (178 to 372 g / kg; Table 1) and high DMED values (Table 2), as discussed later. Samples of cultures' contents collected at the end of the 24 h fermentations in sterile containers were freeze-dried, pooled by seaweed (within each run), and 50 mg were homogenized with steel beads (2.3 mm) in a Mini-Bead-beater 8 (BioSpec Inc, Bartlesville, OK; USA) before DNA extraction following the repeated bead beating plus column (RBB+C) procedure described by Yu and Morrison (2004). The OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA) was used to remove PCR inhibitors and the QIAmp® DNA Stool Mini Kit columns (QIAgen, Valencia, CA) were used to purify the DNA. The

yield and purity of the extracted DNA were assessed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The DNA samples were used as templates for quantifying the copy numbers of 16S rRNA (for bacteria), the methyl coenzyme M reductase A (mcrA) gene (for methanogenic archaea), and 18S rRNA (for protozoa) through quantitative PCR (qPCR) as described by Martínez-Fernández et al. (2015), with some modifications in the cycling conditions. Primers used for total bacteria have been described by Maeda et al. (2003) and those for protozoa and methanogenic archaea have been described by Sylvester et al. (2004) and Denman et al. (2007), respectively. Cycling conditions were 95ºC for 5 min, followed by 40 cycles at 95ºC for 15 s, 60ºC for 30 s, 72ºC for 55 s and 75ºC for 6 sec, and final extension at 95ºC for 1 min and 75ºC for 1 min. Melting curves were obtained by 81 cycles at 55 °C for 10 sec. The qPCR analyses were performed in triplicate with an iQ5 multicolor qPCR Detection System (BioRad Laboratories, Hercules, CA, USA). The absolute amount of DNA for each microbial group, expressed as the number of DNA copies per gram of fresh matter, was determined with standards. The standards are described in detail in Martínez-Fernández et al. (2015). The absolute amounts of each group of microorganisms were expressed as the corresponding gene copies per g fresh matter sample, and to attain normality, data on gene copies per g fresh matter were transformed using $\log_{10}$ before statistical analysis.

For PCR-DGGE analysis of total bacteria, the V3 region of the 16S rRNA gene was amplified as described by Abecia et al. (2014) using the bacterial primers described by Muyzer et al. (1993). For PCR-DGGE analysis of archaeal community, the mcrA gene was amplified as described by Abecia et al. (2012) using the primers described by Cheng et al. (2009). For the protozoa community, the 18S rRNA was amplified using the primers described by Regensbogenova et al. (2004) and following the procedure of

De la Fuente et al. (2009). The DGGE analyses were performed using the BDH system from VWR International Ltd (UK). The DNA was visualised by silver staining with a Bio-Rad Silver stain kit and scanned DGGE images were analysed with the Quantity One Software (BioRad, Madrid, Spain) by scoring for the presence or absence of bands at different positions in each line. The Shannon's diversity index was used to evaluate the diversity of bacterial communities, and dendrograms were constructed using the percent similarity and unweighted pair-group method using arithmetic averages (UPGMA) options in the MVSP v3.12d software (Kovach Computing Service, Anglesey, Wales, UK).

2.5. Chemical analyses

Procedures for analysis of DM, ash, CP and NDFom in seaweed species are detailed in Tayyab et al. (2016). Briefly, DM concentration was estimated as freeze dry matter and ash was determined as residue after combustion at 525ºC. The CP values were calculated as N × 6.25 after N analysis by the Kjeldahl method. The aNDFom content was measured using FibertecTMM6 system (Foss Analytical, Hillerød, Denmark) using heat stable amylase and sodium sulphite according to the procedure described by Mertens (2002) and expressed exclusive of residual ash. Total extractable polyphenols (TEP) content was analyzed according to Julkunen-Tiito (1985). Ether extract content was determined by ether extraction (AOAC, 2005). The amount of non-structural carbohydrates (NSC) was calculated as [1000 – (ash + CP + aNDFom + EE)], all expressed as g/kg DM.

Individual VFA concentrations were analyzed by gas chromatography described by Isac et al. (1994), using an Autosystem Perkin-Elmer Cor., Norwolk, CT) equipped with a crosslinked 100 % polyethylene glycol column (TRB-FFAP, 30 m × 0.53 mm i.d. × 1µm film thickness, Teknokroma, Madrid, Spain). Methane concentration was

determined by gas chromatography using a HP Hewlett 5890 Packard Series II gas chromatograph (Waldbronn, Germany) equipped with a flame ionization detector (FID) and an HP-INNOWAX cross linked polyethylene glycol column (25 m x 0.2 mm x 0.2 μm; Teknokroma, Madrid, Spain). The carrier gas was N_2 and peaks were identified by comparison with a standard of known composition. A sample of 0.5 ml of gas was injected using a 1 ml Sample-Lock® syringe (Hamilton, Reno, NV, USA). The amount of methane produced in each culture was calculated by multiplying the total gas produced by the concentration of methane obtained.

2.6. Calculations and statistical analysis

Gas production data were fitted to the exponential model: $gas = A\ (1 - e^{(-c\ (t-lag))})$, where A is the asymptotic gas production, *c* is the fractional rate of gas production, *lag* is the initial delay in the onset of gas production, and t is the time of gas measurement. The parameters A, *c* and *lag* were estimated by an iterative least squares procedure using the NLIN procedures of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA). The average gas production rate (AGPR; mL gas/h) is defined as the average gas production rate between the start of the incubation and $T_{1/2}$, and was calculated as $AGPR = A\ c\ /\ [2\ (\ln 2 + c\ lag)]$. Finally, the DM effective degradability (DMED) was estimated assuming a rumen particulate outflow (K_p) of 0.03 per h according to the equation: $DMED = [\ (DMD_{72}\ c)\ /(c + K_p)]\ e^{(-c\ lag)}$. The amount of VFA in each batch culture after 24 h of incubation was corrected for the amount of VFA in the rumen fluid used as inoculum.

Within each incubation run, the values measured in the two bottles incubated for 24 or 72 h for each seaweed sample were averaged before statistical analysis (three replicates per sample). All data were analyzed as a mixed model using the PROC MIXED of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA) with seaweeds species, season and seaweeds species x season interaction as fixed effects and incubation run as

random effect. When a significant effect of seaweeds species ($P \leq 0.05$) was detected, differences among means were tested using the Tukey's multiple comparison test. Relationships between chemical composition and DM degradability and gas production parameters were investigated by linear regression using the PROC CORR of SAS (SAS Inst. Inc., Cary, NC, USA).

3. Results

3.1. Chemical composition

Chemical composition of seaweeds was affected by both seaweed species and collecting season, with the exception of DM content (Table 1). In general, brown seaweeds had greater ash concentrations compared with red and green species. The CP content varied widely, with *Porphyra* and *Acrosiphonia* having the greatest ($P < 0.05$) contents and all brown species showing values lower than 145 g/kg DM. *Alaria*, *Laminaria* and *Mastocarpus* had aNDFom concentrations lower than 260 g/kg DM, whereas *Palmaria* had values above 450 g/kg DM. *Pelvetia* had the greatest ($P < 0.001$) EE content and *Porphyra sp.* and *Palmaria* had the lowest contents. The NSC content varied from 135 to 541 g/kg DM (values averaged across seasons), with brown seaweeds having the greatest values. Ash and CP contents were higher in spring than in autumn ($P = 0.020$ and 0.003, respectively), whereas concentrations of aNDFom, EE and NSC were not affected by collecting season ($P = 0.208$ to 0.341). Total extractable polyphenols (TEP) content ranged from 1.46 (*L. digitata*, spring sample) to 50.3 (*A. esculenta*, autumn sample) mg/g DM, and differed among seaweed species ($P < 0.001$) and collecting season ($P < 0.001$). *Alaria esculenta* and *Pelvetia canaliculata* had higher ($P < 0.05$) TEP contents than the rest of the seaweeds in both seasons. Contents of TEP in the samples collected in autumn were higher ($P < 0.001$) than those in collected in spring.

3.2. Gas production kinetics

The parameters of gas production kinetics of the seaweeds are presented in Table 2. Seaweed species × season interactions were detected for A (P = 0.002), AGPR (P < 0.001), DMED (P < 0.001), and $TDMD_{72}$ (P < 0.001). There were differences (P < 0.001) among seaweeds species in all the gas production parameters measured, as well as in the DMED and $TDMD_{72}$. In contrast, season did not affect (P > 0.05) the values of A and *lag*, but autumn-samples had greater values (P < 0.001) of *c* (0.092 vs. 0.072 per h), AGPR (3.20 vs. 2.18 mL / h) and DMED (543 *vs.* 522 g / kg) compared with spring-samples. The highest (P < 0.05) values for A and AGPR were observed for *Palmaria palmata* (123 ml 6.20 ml / h, respectively; mean across seasons) and the lowest ones for *Pelvetia canaliculata* (10.2 ml and 0.71 ml / h, respectively). *Mastocarpus stellatus* had the highest (P < 0.05) gas production rate (0.119 per h, mean across seasons), although the difference with *Pelvetia canaliculata* was not significant (P > 0.05). The *lag* time was 0.00 for most of the seaweeds, with the exception of *Pelvetia canaliculata*, *Palmaria palmata* and *Porphyra sp.* (0.31, 1.19 and 0.05 h, respectively). The DMED values ranged from 424 and 652 g / kg, and were higher (P < 0.05) for the three red seaweeds (*Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra sp.*) than for the rest of seaweeds. *Acrosiphonia sp.* and *Pelvetia canaliculata* had the lowest (P < 0.05) $TDMD_{72}$ values, whereas *Laminaria digitata* and *Palmaria palmata* had the greatest values.

3.3. In vitro *rumen fermentation characteristics*

A period of 24 h of incubation was selected for assessing ruminal fermentation parameters, because it corresponds to a rumen passage rate of 0.041 per h, which can be found in sheep consuming fibrous diets (Ranilla et al., 1998). There were differences among seaweed species in both total VFA production and profile (Table 3). *Palmaria*

palmata had the highest ($P < 0.05$) VFA production, whereas *Mastocarpus stellatus* and *Pelvetia canaliculata* had the lowest values ($P < 0.05$). Seaweeds collected in autumn had lower VFA production compared with those collected in spring, and no seaweed species × season interaction ($P = 0.213$) was detected. The molar proportion of acetate was not affected either by the seaweed species ($P = 0.408$) or the season ($P = 0.407$). In contrast, molar proportion of the rest of VFA differed among seaweed species, with *Palmaria palmata* having the highest proportion of propionate and the lowest ($P < 0.05$) proportion of butyrate, these last values being similar ($P > 0.05$) to those for *Pelvetia canaliculata*, *Acrosiphonia sp.* and *Porphyra sp.* There was a high variability in the acetate:propionate ratio, which ranged from 2.16 to 4.81, with *Palmaria palmata* showing the lowest value ($P < 0.05$) and *Pelvetia canaliculata* the highest ($P < 0.05$). Significant seaweed species × season interactions ($P < 0.05$) were observed for the molar proportions of acetate, propionate, butyrate and valerate, as well as for the acetate:propionate ratio.

Both seaweed species and season affected ($P < 0.05$) final pH values in the fermentation medium, ammonia-N concentrations and methane production (Table 4), but no seaweed species × season interactions were detected for these parameters (P = 0.851, 0.995 and 0.178, respectively). *Palmaria palmata* had the lowest ($P < 0.05$) pH values and the highest ($P < 0.05$) methane production. Higher ($P < 0.05$) pH values and lower ($P < 0.05$) values of ammonia-N concentrations and methane production were observed for the samples collected in autumn compared with those collected in spring. In contrast, the ratio methane to total VFA was not affected ($P > 0.05$) by either the season or seaweed species.

3.4. Microbial abundance and diversity in cultures with red seaweeds

The microbial concentrations and diversity of total bacteria, protozoa and methanogenic archaea in the cultures with *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra sp.* collected in spring as substrates is presented in Table 5. There were no differences in the abundance of total bacteria, archaea and protozoa (P = 0.922, 0.303 and 0.367, respectively) among the cultures with the three red seaweeds. The numbers of bands in the DGGE gels of individual samples ranged from 29 to 61 for bacteria, from 10 to 16 for archaea, and from 5 to 8 for protozoa. No differences (P = 0.230 to 0.444) among seaweeds were detected either in the number of bands or in the Shannon index.

Figure 1 shows the dendrograms of the DGGE profiles of *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra sp.* samples after 24-h *in vitro* incubations in batch cultures. For all microbial populations analyzed, samples were separated by the incubation run and formed three clear clusters. The DGGE profiles of *Mastocarpus stellatus* and *Porphyra sp.* in the incubation runs 1 and 3 showed a similarity of 100% for archaea and greater than 96% for bacteria, but values in the incubation run 2 were lower (95 and 86% for archaea and bacteria, respectively). The three seaweeds showed a similarity of 100% for protozoa in the incubation runs 1 and 3, but *Palmaria palmata* had a lower similarity (90%) with *Mastocarpus stellatus* and *Porphyra sp.* in the incubation run 2. No clear grouping of samples by seaweed species were observed for any of the microbial population analyzed.

4. Discussion

4.1. Chemical composition, gas production kinetics and in vitro *rumen fermentation characteristics*

The influence of seaweed species and collecting season on DM, ash, CP and aNDFom content has been discussed by Tayyab et al. (2016). Ether extract content was

within the range reported in the literature for these seaweeds (Dawczynski et al., 2007; Makkar et al., 2016), with values below 30 g/kg DM for all samples, which are lower than the value above that the growth of cellulolytic bacteria can be reduced (50 g/kg DM; Doreau and Chilliard, 1997). However, the negative relationship between EE content and both DMED (r = 0.628; *P* = 0.016) and DMD_{72} (r = 0.742; *P* = 0.002) may indicate a negative effect of fat on *in vitro* ruminal degradation of seaweeds. In fact, the seaweeds with the lowest fat content (*Mastocarpus* and *Porphyra sp.*) had the greatest DMED values (see Table 2), whereas those with greater EE content (*Pelvetia* and *Acrosiphonia sp.*) showed the lowest DMED values. This point deserves further investigation. In contrast, EE content was not related (*P* > 0.05) to either total VFA production or any of the gas production kinetics parameter (A, *c*, *lag* and AGPR). In general, brown seaweeds had greater NSC contents than red and green seaweeds in our study, which is consistent with the lower aNDFom observed in the brown seaweeds. Neither aNDFom nor NSC contents were related (*P* < 0.05) to DMED, DMD_{72} or gas production kinetics parameters, which reflects the complexity of the compounds included in aNDFom and NSC fractions.

As previously reported by others (Belanche et al., 2016a; Makkar et al., 2016), there were marked differences among seaweeds in their TEP content. In general, the brown seaweeds had higher TEP contents than red and green seaweeds in our study, and those collected in spring had lower values than those sampled in autumn. Polyphenols in plants can protect proteins from degradation and improve the efficiency of use of nitrogen in ruminants by increasing the amount of by-pass protein, but can also reduce the rumen fibre degradation by decreasing the attachment of microbes to feed particles (Makkar, 2003). The negative relationships between the TEP content and the DMED (r = 0.544; P = 0.044) and $TDMD_{72}$ (r = 0.602; P = 0.023) values, observed in the present

study indicate a negative effect of TEP on *in vitro* rumen degradation of seaweeds. In contrast, no relationship ($P > 0.05$) was detected between the TEP content and any of the gas production kinetics parameters. As pointed out by Makkar (2003), gas measurements should be combined with determinations of microbial growth and/or feed degradability for a better interpretation of the effects of polyphenols.

There were important differences among seaweed species in their DMED, with *Mastocarpus stellatus* and *Porphyra sp.* showing the highest average values (> 600 g / kg) and *Acrosiphonia sp.* and *Pelvetia canaliculata* the lowest (453 and 444 g / kg, respectively). Tayyab et al. (2016) using a *in situ* technique in dairy cows and the same seaweeds samples than those used in the present work found lower DM degradability values for *Pelvetia canaliculata* and *Mastocarpus stellatus* (246 and 270 g / kg, respectively) and higher for *Palmaria palmata* (833 g / kg; values at 24 h incubation averaged across seasons) compared to the DMED values found in the present work. The differences in the results might be due to the use of different methodology (*in vitro vs. in situ*) and animal species (sheep *vs.* cows). However, the 24-h *in situ* degradability data determined by Tayyab et al. (2016) agree well with the VFA production measured after 24 h *in vitro* incubation in our study, with *Palmaria palmata* having the highest values (4.34 mmol of VFA and 833 g/kg for *in situ* DM degradability) and *Pelvetia canaliculata* and *Mastocarpus stellatus* having the lowest values for both parameters (1.85 and 1.84 mmol of VFA, respectively, and 245 and 270 g/kg DM for *in situ* DM degradability). However none of the chemical constituents determined was correlated ($P > 0.05$) with total VFA production, with the exception of EE content and a trend to a negative correlation between the TEP content and the amount of total VFA produced ($r = 0.470$; $P = 0.090$).

Seaweeds differed in their VFA profile, with *Palmaria palmata* having the highest propionate proportions (23.0 and 26.0 mol / 100 mol in spring and autumn, respectively) and the lowest acetate/propionate ratio (2.41 and 2.28). Conversely, *Pelvetia canaliculata* and *Mastocarpus stellatus* had the highest acetate/propionate ratios (4.29 and 4.48, and 3.85 and 4.17, respectively). High variations between seaweed species in the VFA profile in *in vitro* fermentations have also been reported by others (Kinley et al., 2016; Machado et al., 2016). Branched-chain VFA are generated from the degradation of some amino acids and their concentrations in *in vitro* cultures can be used as an index of protein degradation. *Mastocarpus stellatus*, *Porphyra sp.* and *Acrosiphonia sp*. had the highest molar proportions of isoacids (6.63, 6.61 and 5.80 mol / 100 mol, respectively; calculated as isobutyrate plus isovalerate), which is in agreement with the high ammonia-N values (29.2, 46.1 and 40.9 mg / 100 ml) observed in the cultures of these seaweeds and with the high CP content of these seaweeds (see Table 1), specially *Porphyra sp.* and *Acrosiphonia sp*. *Alaria esculenta* showed the lowest isoacids proportion and ammonia-N concentration (3.56 mol / 100 mol and 18.9 mg / 100 ml), despite its CP content was numerically greater than that of *Laminaria* and *Pelvetia* (see Table 1). Interpretation of isoacids proportions is difficult, as they are captured and used by the cellulolytic bacteria for their growth (Hume, 1970).

Several studies have investigated the effects of including seaweeds in the diet of ruminants on methane emissions, but results have been contradictory. Belanche et al. (2016b) observed no changes in methane production in Rusitec fermenters by including either one of the brown seaweed *Laminaria digitata* or *Ascophyllum nodosum* at 50 g / kg in the diet (DM basis). In contrast, Kinley et al. (2016) and Machado et al. (2016) observed that the red seaweed *Asparagopsis taxiformis* was highly effective in decreasing methane production in batch cultures at doses as low as 20 g / kg (organic

matter basis), but higher doses also decreased VFA production. Machado et al. (2016) also reported an antimethanogenic effect of the freshwater green filamentous alga *Oedogonium sp.* at higher doses (> 500 g/kg organic matter). In our study, the positive relationship (r = 0.943; P = <0.001) observed between the amount of total VFA and methane production in the cultures indicates that methane production was directly related to the amount of substrate fermented, as both VFA and methane are produced in the fermentation of organic matter. This positive relationship might also indicate an absence of antimethanogenic compounds in any of the tested seaweeds, which is supported by the lack of differences between seaweeds in methane / total VFA ratio. Morever, the values of fermentation parameters (VFA, NH_3-N and methane) were in the range of those obtained in previous studies by our group in batch cultures of sheep rumen microorganisms containing feeds commonly used in ruminants feeding (Martinez et al., 2010; Soto et al., 2015; Mateos et al., 2015, 2016), which would indicate that the tested seaweeds do not contain compounds inhibiting the growth of ruminal microorganisms. It should be noticed that in our study the diet fed to donor sheep did not contain seaweeds, and adaptation to seaweeds consumption might have changed the rumen microbial populations (Belanche et al., 2016b) and therefore the results obtained. In a recent review on the use of *in vitro* systems, Yañez-Ruiz et al. (2016) indicated that the effects of differences in diet composition fed to donor animals may be minimized by obtaining rumen fluid immediately before feeding, and this approach was followed in the present study.

The differences between seasons, with spring-samples having higher DMED values and VFA production compared with autumn-samples, are in accordance with the results of Tayyab et al. (2016) for the same batch of seaweed samples. This would indicate that spring-collected seaweeds had higher nutritive value than those collected in autumn for

both sheep and cows. These results are consistent with the lower CP content and higher aNDFom content for the autumn-seaweeds compared with the seaweeds collected in spring (see Table 1). Autumn-collected brown seaweed had higher TEP and are therefore of more interest when looking for bioactive effects from the seaweeds. However, a species × season interaction was detected in some of the parameters analyzed in our study, indicating differences in season effect among species. Moreover, other parameters such as the habitat and external conditions (water temperature and movement, sunlight intensity, nutrient concentration in water, etc.) might influence the chemical composition and nutritive value of seaweeds (Makkar et al., 2016).

4.2. Microbial abundances and diversity

The microbial analysis in the cultures could be conducted only in a limited number of samples due to economic and technical constraints. The three red seaweeds (*Mastocarpus estellatus*, *Palmaria palmata* and *Porphyra sp.*) collected in spring were selected for these analyses due to their relatively high CP content and high DMED values (Tables 1 and 2, respectively), and therefore their potential as feed ingredients in ruminant diets. These seaweeds differed markedly in their AGPR (1.50 to 4.55 mL / h), methane (0.567 to 981 mmol) and VFA production (2.79 to 5.02 mmol), as well as VFA profile (acetate:propionate ratios from 2.41 to 3.85). These variable fermentation patterns might indicate the existence of differences in the microbial populations promoted by these seaweeds. Contrary to our expectations, no significant differences in the abundance of any of the analysed microbial populations were detected. However, the abundance of bacteria in the cultures with *Palmaria palmata* was 20.6 and 10.8 % higher than that for *Mastocarpus stellatus* and *Porphyra sp.*, respectively, which is consistent with the greater fermentability of *Palmaria palmata,* as indicated by its high VFA and methane production values (5.02 and 0.981 mmol, respectively).

The lack of differences between the three red seaweeds in the abundance of archaea contrasts with the variability observed in methane production in the same cultures (0.584, 0.981 and 0.567 mmol for *Mastocarpus stellatus, Palmaria palmata* and *Porphyra sp.*, respectively). In agreement with other studies (Machmüller et al., 2003; Yáñez-Rúiz et al., 2008; Mateos et al., 2016), there were no relationship between the abundance of methanogenic archaea and the methane production (r = 0.333; *P* = 0.421; n = 9), indicating that there is no clear relationship between the number of methanogenic archaea and methanogenesis in the rumen.

The abundances of protozoa were similar to the values ranging from 8.24 to 8.66 (log gene copies / g fresh matter) reported by Soto et al. (2015) in an experiment with batch cultures containing four mixed diets (57:43 forage:concentrate) as substrates and conducted with the same *in vitro* and DGGE methodologies used in the present study. These results indicate that none of the tested red seaweeds had antiprotozoal compounds. In agreement with our results, Belanche et al. (2016b) also observed no effect of the 5% inclusion of two brown seaweeds (*Laminaria digitate* and *Ascophyllum nodosum*) in the diet on protozoa abundances in Rusitec fermenters, but Boeckaert et al. (2007) reported a decrease in the abundance of some protozoa in the rumen of dairy cows by feeding a unicellular fungi (*Schizochytrium sp.*; 2 % of intake). It seems that different seaweed species can have variable effects on rumen microbial populations.

The number of DGGE-bands for bacteria (20 to 61) was similar to that previously reported in sheep and batch cultures (Belanche et al., 2012; Saro et al., 2014) using the same primers utilized in our study. The number of bands observed in the DGGE gels for protozoa (5 to 8) is consistent with the results of De la Fuente et al. (2009), who observed up to 8 bands in the rumen of goats using the same DGGE methodology utilized in our study. The lack of differences in the number of bands in the DGGE gels

and in the values of Shannon index indicates a similar diversity of bacteria, archaea and protozoa in the cultures with *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra sp.* as substrates. The cluster pattern observed in the dendrograms of DGGE profiles indicates that the incubation run (inoculum) had the largest effect on all analysed microbial populations, as previously reported by others both in batch cultures (Mateos et al., 2015; 2016) and in the rumen of sheep (Saro et al., 2012; 2014). Although the rumen fluid used as inoculum was obtained from sheep that received the same standard diet during the *in vitro* experiments and rumen content was sampled immediately before feeding the three clear clusters formed according incubation run indicate differences in microbial populations among the rumen fluids used as inoculum in each incubation runs. Mateos et al. (2015) analyzed the changes in bacterial diversity over the *in vitro* incubation in batch cultures containing four different diets and inoculated with fluid from sheep fed the same diets, and observed that each batch culture sample showed the highest similarity index with its corresponding rumen inoculum. The results of the present study show the influence of the microbial populations in the inoculum on the microbial populations promoted by the seaweeds.

5. Conclusions

There were marked differences between the tested seaweed species in their rate and extent of rumen fermentation. The collection season significantly affected most of the analyzed parameters, and the seaweeds collected in spring had higher protein content and higher *in vitro* rumen degradability and lower total extractable polyphenols content compared with those collected in autumn. As indicated by the volatile fatty acid production, *Palmaria palmata* had the highest degradability in 24-h incubations, whereas *Pelvetia canaliculata* and *Mastocarpus stellatus* had the lowest. Further *in vivo* studies are required to identify the factors affecting the nutritive value of seaweeds for

ruminants and to determine the optimal seaweed inclusion levels in the diets for ruminants.

The authors of the manuscript entitled "*In vitro* ruminal fermentation and methane production of different seaweed species" declare that there are no conflicts of interest.

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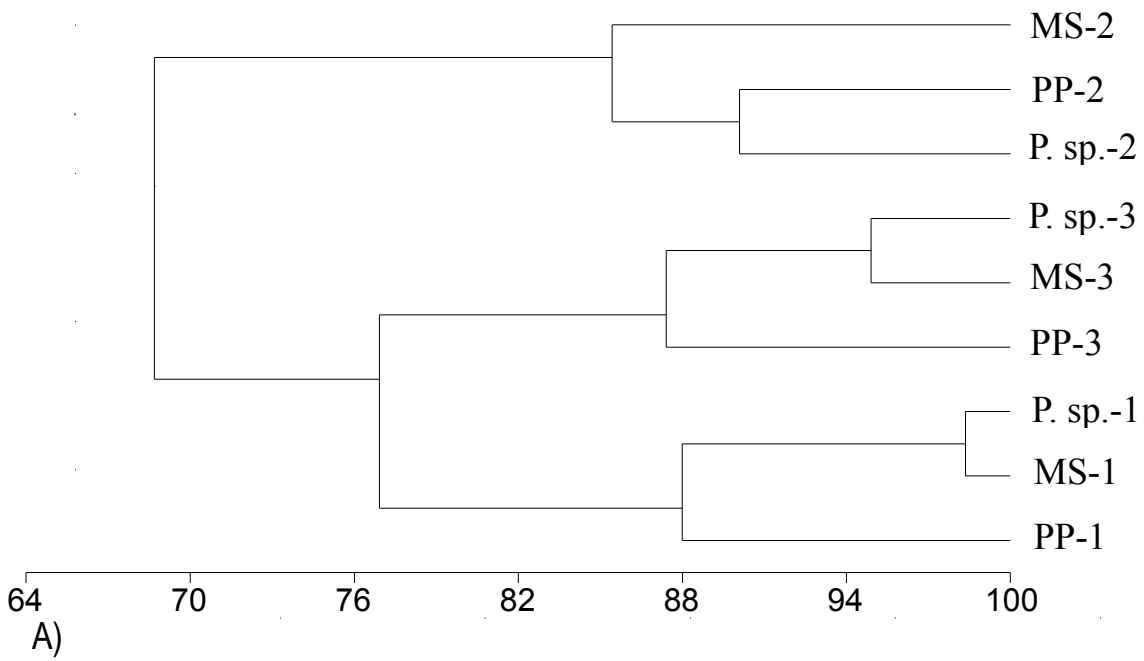

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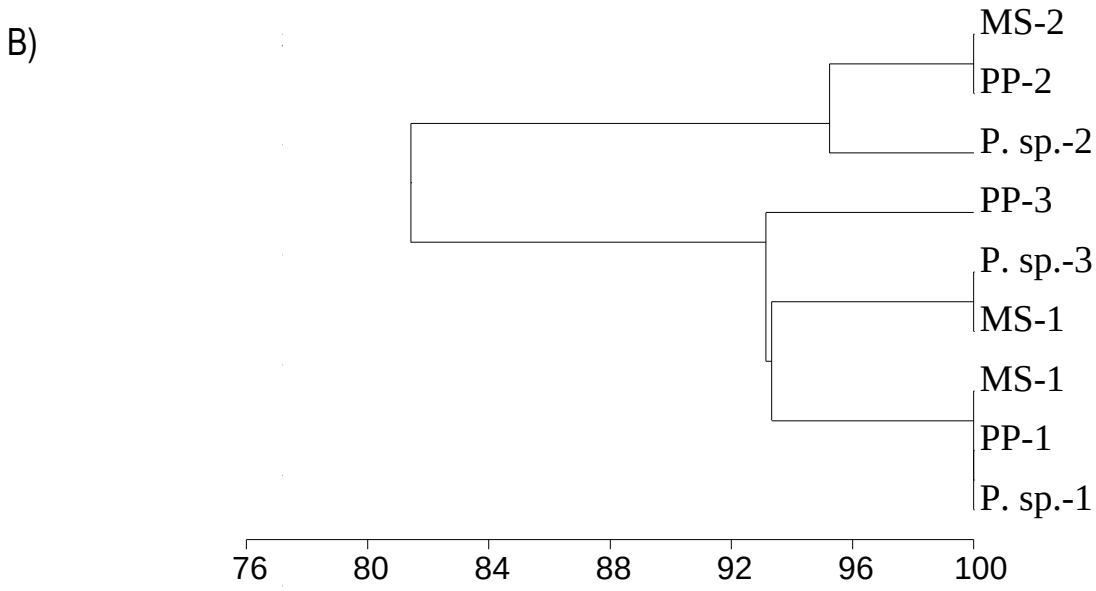

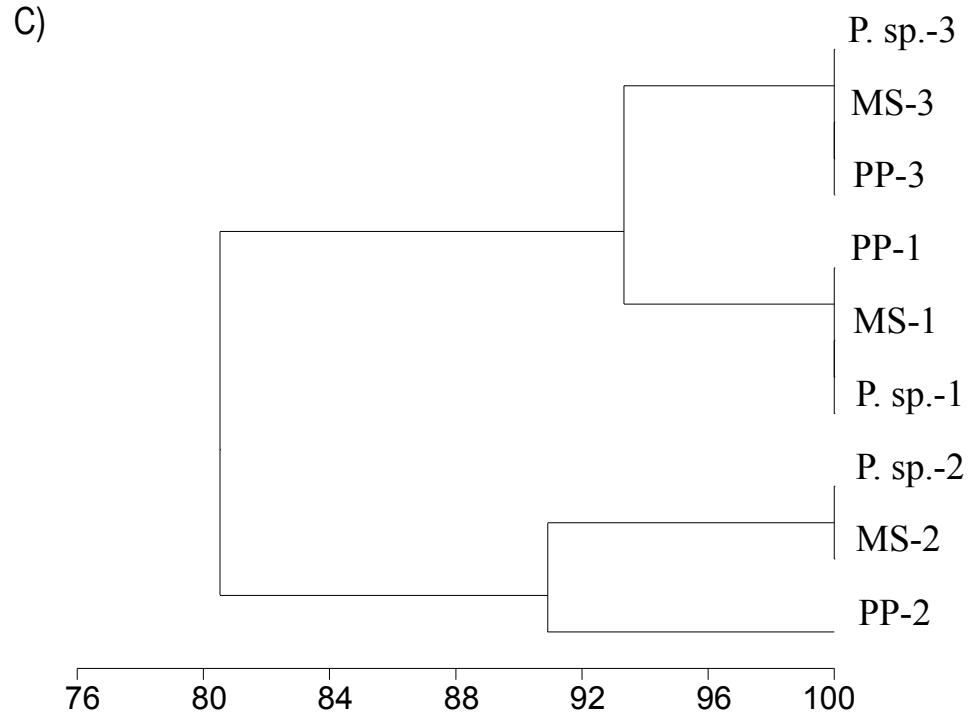

Figure 1. Dendrograms of DGGE analysis of total bacteria (Figure 1A), archaea (Figure 1B) and protozoa (Figure 1C) communities in 24-h *in vitro* ruminal fermentations of seaweeds from northern Norway. MS: *Mastocarpus stellatus;* PP: *Palmaria palmata;* P. sp.: *Porphyra sp.* Numbers 1 to 3 correspond to incubation runs. Scale bars show percentage of similarity.

Table 1. Chemical composition (mg / g DM, excepting DM content) in different seaweed species collected during spring and autumn in northern Norway[1]

Seaweed species	Season	DM	Ash	CP	EE	NSC	TEP
Brown seaweeds							
Alaria esculenta	Spring	132	278	158	4.24	443	8.91
	Autumn	237	139	127	4.92	639	50.3
	Average	**185**	**209^{ab}**	**143^{ab}**	**4.58^{c}**	**541^{c}**	**29.6^{b}**
Laminaria digitata	Spring	128	351	161	3.35	322	1.46
	Autumn	173	233	103	3.48	460	2.25
	Average	**151**	**292^{b}**	**132^{ab}**	**3.42^{bc}**	**391^{c}**	**1.86^{a}**
Pelvetia canaliculata	Spring	229	219	105	28.4	355	18.8
	Autumn	244	210	75.0	27.9	407	35.7
	Average	**237**	**215^{a}**	**90.0^{a}**	**28.2^{e}**	**381^{bc}**	**27.2^{b}**
Red seaweeds							
Mastocarpus stellatus	Spring	283	217	178	0.564	456	3.44
	Autumn	254	208	178	0.583	262	4.21
	Average	**269**	**213^{a}**	**178^{bc}**	**0.574^{a}**	**359^{bc}**	**3.82^{a}**
Palmaria palmata	Spring	160	165	257	3.33	154	3.97
	Autumn	200	108	188	1.63	201	2.59
	Average	**180**	**137^{a}**	**223^{c}**	**2.48^{b}**	**178^{ab}**	**3.28^{a}**
Porphyra sp	Spring	148	149	372	0.458	108	3.64
	Autumn	105	107	321	0.823	163	4.76
	Average	**127**	**128^{a}**	**347^{d}**	**0.641^{a}**	**135^{a}**	**4.20^{a}**
Green seaweeds							
Acrosiphonia sp	Spring	226	171	333	7.63	82.4	3.88
	Autumn	194	127	286	6.09	193	5.17
	Average	**210**	**149^{ab}**	**310^{d}**	**6.86^{d}**	**138^{a}**	**4.52^{a}**
P value							
Species		0.093	0.003	<0.001	<0.001	0.021	<0.001
Season		0.171	0.020	0.003	0.341	0.261	<0.001
SEM		39.9	35.8	16.1	0.658	87.5	0.368

$^{a, b, c, d, e}$ Average values for each seaweed not sharing the same superscript differ ($P < 0.05$)

[1] Data on DM, ash, CP and aNDFom content were reported previously by Tayyab et al. (2016). DM: dry matter (g/kg); CP: crude protein; aNDFom: ash free neutral detergent fiber; EE: ether extract; NSC: non-structural carbohydrates (calculated as 1000 – (ash + CP + aNDFom + EE); TEP: total extractable polyphenols.

Table 2. *In vitro* gas production kinetics, dry matter effective degradability (DMED) and true DM digestibility after 72 h of incubation ($TDMD_{72}$) of different seaweed species collected during spring and autumn in northern Norway.

		Parameters of gas production kinetics[1]					
Seaweeds species	Season	A (ml)	c (per h)	Lag (h)	AGPR (mL / g DM)	DMED (g / kg)[2]	$TDMD_{72}$ (g / kg)
Brown seaweeds							
Alaria esculenta	Spring	64.8	0.047	0.00	2.32	501	814
	Autumn	51.1	0.063	0.00	2.13	449	664
	Average	**58.0^{d}**	**0.055^{a}**	**0.00^{a}**	**2.23^{c}**	**475^{ab}**	**739^{b}**
Laminaria digitata	Spring	93.7	0.035	0.00	2.38	483	895
	Autumn	79.8	0.057	0.00	3.27	557	852
	Average	**86.8^{e}**	**0.046^{a}**	**0.00^{a}**	**2.83^{c}**	**520^{b}**	**874de**
Pelvetia canaliculata	Spring	11.9	0.096	0.00	0.73	463	602
	Autumn	8.58	0.123	0.61	0.69	424	559
	Average	**10.2^{a}**	**0.111^{cd}**	**0.31^{a}**	**0.71^{a}**	**444^{a}**	**581^{a}**
Red seaweeds							
Mastocarpus stellatus	Spring	18.8	0.113	0.00	1.50	652	829
	Autumn	15.9	0.115	0.00	1.40	639	858
	Average	**17.4^{a}**	**0.119^{d}**	**0.00^{a}**	**1.45^{b}**	**646^{d}**	**844^{d}**
Palmaria palmata	Spring	118	0.060	1.48	4.55	525	860
	Autumn	127	0.096	0.89	7.85	640	914
	Average	**123^{f}**	**0.078^{b}**	**1.19^{b}**	**6.20^{d}**	**583^{c}**	**887^{e}**
Porphyra sp	Spring	31.9	0.093	0.09	2.05	588	787
	Autumn	41.8	0.097	0.00	3.26	629	821
	Average	**36.9^{b}**	**0.095^{bc}**	**0.05^{a}**	**2.66^{c}**	**609^{d}**	**804^{c}**
Green seaweeds							
Acrosiphonia sp.	Spring	45.2	0.059	0.00	1.75	443	627
	Autumn	55.0	0.096	0.00	3.79	463	610
	Average	**50.1^{c}**	**0.077^{b}**	**0.00^{a}**	**2.80^{c}**	**453^{a}**	**619^{a}**
P value							
Species		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Season		0.301	< 0.001	0.928	< 0.001	< 0.001	0.091
Species x Season		0.002	0.337	0.353	< 0.001	< 0.001	< 0.001
SEM		1.01	0.0029	0.066	0.093	7.1	5.0

[a - f]: Within each column, average values for each seaweed not sharing the same superscript differ ($P < 0.05$)

[1] A: asymptotic gas production; *c*: fractional rate of gas production; Lag: initial time delay in the onset of gas production; AGPR: average gas production rate;

[2] DMED: Dry matter effective degradability for a rumen particulate outflow of 0.03 / h

[3] Calculated as described by Van Soest et al. (1966)

Table 3. Total volatile fatty acids (VFA), molar proportions of individual VFA and acetate:propionate ratios (Ac / Pr) after 24-h *in vitro* incubation of seaweeds of different seaweed species collected during spring and autumn in northern Norway in batch cultures of ruminal microorganisms.

			Molar proportions (mol / 100 mol)						
Seaweed species	Season	VFA (mmol)	Ac	Pr	But	Isobut	Isoval	Val	Ac / Pr (mol / mol)
Brown seaweeds									
Alaria esculenta	Spring	3.48	60.4	15.9	17.1	1.50	3.03	2.10	3.84
	Autumn	1.35	51.4	21.7	19.0	1.16	1.42	5.55	2.37
	Average	**2.42^{a}**	**55.9**	**18.8^{b}**	**18.1^{b}**	**1.33^{ab}**	**2.23^{a}**	**3.83^{d}**	**3.08^{b}**
Laminaria digitata	Spring	3.14	64.6	13.4	14.1	1.76	4.11	2.00	4.81
	Autumn	1.78	55.2	21.2	17.1	1.54	2.46	2.15	2.62
	Average	**2.46^{a}**	**59.9**	**17.3^{b}**	**15.6^{ab}**	**1.65^{bc}**	**3.29^{bc}**	**2.05^{a}**	**3.71^{bc}**
Pelvetia canaliculata	Spring	2.97	57.7	13.5	21.4	1.62	3.67	2.15	4.29
	Autumn	0.73	59.6	14.1	16.2	1.51	1.80	6.94	4.27
	Average	**1.85^{a}**	**58.7**	**13.8^{a}**	**18.8^{b}**	**1.57^{ab}**	**2.74^{ab}**	**4.55^{e}**	**4.25^{d}**
Red seaweeds									
Mastocarpus stellatus	Spring	2.79	55.2	14.4	21.1	2.10	4.68	2.51	3.85
	Autumn	0.88	62.5	15.2	11.9	2.47	4.41	4.70	4.17
	Average	**1.84^{a}**	**58.9**	**14.8^{a}**	**16.5^{ab}**	**2.29^{cd}**	**4.55^{c}**	**3.61^{cd}**	**3.97^{cd}**
Palmaria palmata	Spring	5.02	55.4	23.0	15.1	1.31	2.57	2.65	2.41
	Autumn	3.66	59.3	26.0	9.22	1.13	1.62	2.81	2.28
	Average	**4.34^{b}**	**57.4**	**24.5^{c}**	**12.2^{a}**	**1.22^{a}**	**2.10^{a}**	**2.73^{ab}**	**2.34^{a}**
Porphyra sp	Spring	3.07	58.3	16.8	15.9	2.26	4.63	2.72	3.47
	Autumn	1.80	59.8	18.1	13.7	2.56	3.77	2.82	3.52
	Average	**2.43^{a}**	**59.1**	**17.5^{b}**	**14.8^{a}**	**2.41^{d}**	**4.20^{c}**	**2.77^{b}**	**3.39^{b}**
Green seaweeds									
Acrosiphonia sp	Spring	3.63	58.8	14.1	15.9	1.99	4.36	2.84	4.40
	Autumn	1.83	62.1	20.1	11.5	2.06	3.19	3.04	3.00
	Average	**2.73^{a}**	**60.5**	**17.1^{ab}**	**13.2^{a}**	**2.03^{c}**	**3.78^{bc}**	**2.94^{bc}**	**3.70^{bc}**
P value									
Species		0.033	0.408	< 0.001	0.042	< 0.001	< 0.001	< 0.001	< 0.001

Season		< 0.001	0.407	< 0.001	0.001	0.831	< 0.001	< 0.001	< 0.001
Species x Season		0.981	0.002	< 0.001	0.043	0.268	0.798	< 0.001	< 0.001
SEM		0.213	0.718	0.474	0.713	0.0546	0.180	0.105	0.072

[a, b]: Within each column, average values for each seaweed not sharing the same superscript differ ($P < 0.05$)

[1] Ac: acetate; Pr: propionate; Bt: butyrate; Isobut: isobutyrate; Isovalerate: isovalerate; Val: valerate.

Table 4. Final pH, NH_3-N concentration, methane production, and methane / total volatile fatty acids (methane / VFA) ratio after 24-h *in vitro* incubation in batch cultures of ruminal microorganisms different seaweed species collected during spring and autumn in northern Norway

Seaweed species	Season	pH	NH_3-N (mg 100 ml)	Methane (mmol)	Methane / VFA (mol / mol)	
Brown seaweeds						
Alaria esculenta	Spring	6.70	32.6	0.596	0.181	
	Autumn	6.85	5.21	0.091	0.067	
	Average	**6.78^{b}**	**18.9^{a}**	**0.343^{a}**	**0.119**	
Laminaria digitata	Spring	6.79	42.1	0.467	0.148	
	Autumn	6.90	13.1	0.273	0.154	
	Average	**6.85^{b}**	**27.6^{ab}**	**0.370^{ab}**	**0.151**	
Pelvetia canaliculata	Spring	6.80	34.5	0.477	0.160	
	Autumn	7.00	4.13	0.139	0.190	
	Average	**6.90^{c}**	**19.3^{a}**	**0.308^{a}**	**0.175**	
Red seaweeds						
Mastocarpus stellatus	Spring	6.83	38.7	0.584	0.209	
	Autumn	7.02	19.6	0.11	0.128	
	Average	**6.93^{c}**	**29.2^{ab}**	**0.348^{a}**	**0.168**	
Palmaria palmata	Spring	6.49	41.4	0.981	0.195	
	Autumn	6.64	14.4	0.541	0.148	
	Average	**6.57^{a}**	**27.9^{ab}**	**0.761^{c}**	**0.172**	
Porphyra sp	Spring	6.83	59.6	0.567	0.175	
	Autumn	6.95	32.6	0.445	0.248	
	Average	**6.89^{c}**	**46.1^{c}**	**0.491^{b}**	**0.211**	
Green seaweeds						
Acrosiphonia sp	Spring	6.83	55.2	0.556	0.153	
	Autumn	6.94	26.5	0.312	0.170	
	Average	**6.89^{c}**	**40.9^{bc}**	**0.434^{b}**	**0.162**	
P value						
Species		< 0.001	0.011	0.002	0.873	
Season		< 0.001	< 0.001	< 0.001	0.301	
Species x Season		0.851	0.995	0.178	0.587	
SEM		0.012	2.31	0.0799	0.0471	

[a, b]: Within each column, average values for each seaweed not sharing the same superscript differ ($P < 0.05$)

Table 5. Microbial abundances and diversity (assessed as number of bands (NB) and Shannon-Weimer Index (SI) in DGGE gels) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea (mcrA gene) in 24-h *in vitro* ruminal fermentations of different red seaweed species collected in northern Norway.

	Microbial abundances, Log_{10} (copies / g fresh matter)				Microbial diversity					
					Bacteria		Archaea		Protozoa	
Red seaweeds	Bacteria	Archaea	Protozoa		NB	SI	NB	SI	NB	SI
Mastocarpus stellatus	7.48	4.92	8.04		34.0	3.51	13.7	2.60	6.67	1.88
Palmaria palmata	7.86	6.17	9.51		41.0	3.66	13.7	2.60	7.00	1.94
Porphyra sp.	7.96	6.61	8.89		37.0	3.58	13.3	2.57	6.67	1.87
P value	0.922	0.303	0.367		0.311	0.230	0.444	0.444	0.443	0.443
SEM	0.760	0.596	0.561		2.42	0.044	0.17	0.161	0.167	0.031