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Interspecies and seasonal variations in macroalgae from the Nordic region: Chemical composition and impacts on rumen fermentation and microbiome assembly

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ABSTRACT

Marine macroalgae may serve as sustainable feed resources for ruminant production due to their nutritional attributes, and enteric methane (CH_4) mitigating potential. We aimed to characterize the anti-methanogenic properties of 12 Nordic macroalgae species (eight brown, three red, and one green). Differences in the chemical composition across two harvesting seasons and impacts of addition of macroalgae (20% dry matter basis) on *in vitro* rumen fermentability of maize silage (MS) and associated changes in the rumen microbiome composition were also evaluated. Green and red macroalgae contained twice as much crude protein (CP) as compared to brown macroalgae. The latter had higher mineral and total polyphenol content (TPC: 10 to 20 times). In some brown species, ash and CP contents were up to twice as high in spring than in autumn, but TPC was highest in autumn. The TPC content was inversely correlated with *in vitro* rumen fermentation characteristics: organic matter (OM) degradability ($r = -0.85$; $P < 0.001$), production of total gas ($r = -0.79$; $P < 0.001$), total volatile fatty acids ($r = -0.78$; $P < 0.001$) and CH_4 ($r = -0.53$; $P < 0.03$) per gram of OM. The polyphenol-rich brown species, *Fucus vesiculosus* and *Ascophyllum nodosum*, caused a significant reduction in feed degradability (~25%) due to the suppression of cellulolytic bacteria (*Ruminococcus* spp., *Lacnospiraceae* spp., *Rikenellaceae* RC9 gut group) in the rumen fluid after fermentation. Interestingly, autumn-harvested samples of those two macroalgae decreased the CH_4 production by 62.6% and 48.2%, respectively, and reduced rumen methanogenic archaea (e.g., *Methanobrevibacter* spp.), although the reduction was not directly correlated with TPC. Thus, Nordic macroalgae, depending upon their species-specific unique properties, could be utilized as anti-methanogenic feed additives or feeding resources for ruminants. *In vivo* studies are needed to establish the implications of feeding with these macroalgae on overall animal performance.

1. Introduction

The livestock sector is a critical element of food security worldwide as livestock-based products contribute 13% of calories and 28% of total protein consumed by humans (FAO, 2011). The demand for livestock products is expected to further rise with the growing global population and altered dietary preferences in direction of a higher proportion of animal-derived protein, particularly in developing countries (Alexandratos and Bruinsma, 2012). On the other hand, ruminant livestock such as cattle, sheep, and goats, are major sources of greenhouse gases, and they account for ~18% of the total anthropogenic methane (CH_4) emissions (Mizrahi et al., 2021). Addressing this challenge requires the development of an environmentally friendly, yet productive, livestock sector in the future to fulfill the demands of livestock products without

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increments in the carbon footprint of production. In this regard, changes in feeding strategies can mitigate the greenhouse gas emissions from ruminants (Haque et al., 2014) and various novel feeding materials could play a crucial role in the future to improve the balance between animal productivity and environmental sustainability (Beauchemin et al., 2008).

Marine macroalgae, also called seaweeds, might serve as such alternative feeding materials that can support animal productivity as well as reduce the CH_4 emissions from ruminant livestock (Kinley et al., 2020; Maia et al., 2016). Macroalgae are rich in carbohydrates, dietary fiber, and minerals (Dawczynski et al., 2007) and can contribute to both rumen-degradable as well as bypass protein sources for ruminants (Tayyab et al., 2016). Recent studies have indicated that dietary supplementation of certain macroalgae, such as the tropical red species *Asparagopsis taxiformis*, can reduce CH_4 emissions by targeting the special domain of rumen microbiota responsible for the formation of CH_4, namely the methanogenic archaea (Machado et al., 2018; Roque et al., 2019a). However, the bioactive compounds in *Asparagopsis* spp. responsible for the anti-methanogenic effect are halomethanes, which are ozone degrading, and some are also carcinogenic and may be toxic to animals (Muizelaar et al., 2021). A mild to moderate anti-methanogenic property has also been reported for specific temperate or North Atlantic macroalgae such as *Ascophyllum nodosum* (Belanche et al., 2016b), *Gracilaria vermiculophylla,* and *Ulva* spp. (Maia et al., 2016). However, the responsible anti-methanogenic compounds and their mechanisms of action remain unclear. In a recent study, anti-methanogenic halomethanes, such as bromoform that are found in *Asparagopsis* spp., could not be detected in macroalgae species from the Nordic region (Nørskov et al., 2021). It is therefore highly relevant to clarify if anti-methanogenic properties, potentially associated with safer bioactive compounds, could be detected among Nordic macroalgae species.

Nutritional values, as well as the contents of bioactive compounds in macroalgae, are associated with their phylum (brown, green, or red), genus, and species, and fluctuations can occur across the growing or harvesting seasons (Pandey et al., 2021). Red, as well as green macroalgae, are known to have higher contents of proteins than brown species, but the latter are enriched with higher levels of mineral elements and polyphenols (Molina-Alcaide et al., 2017). In general, protein and minerals are present in the highest concentrations during winter or spring (Rødde et al., 2004; Tayyab et al., 2016), while carbohydrates and polyphenols have been found highest during summer or autumn, but this may vary with the species or type of macroalgae (Connan et al., 2004; Schiener et al., 2015). This seasonal and species-specific variation can affect the digestibility of the algae and the impact on enteric CH_4 production, hence possibilities for utilization as a source of feed or as an anti-methanogenic feed additive for ruminants (de la Moneda et al., 2019; Molina-Alcaide et al., 2017). Information about seasonal and species variation is important to identify the optimal harvest time to achieve maximum nutritional and/or bioactive potential of macroalgae.

Therefore, the current study investigated the interspecies and seasonal variations in the chemical composition of 12 Norwegian macroalgae species, and their impacts on *in vitro* rumen fermentation characteristics, including enteric CH_4 production and composition of the rumen microbiome. Macroalgae selected in this study comprised the dominant wild (e.g., *A. nodosum, Fucus vesiculosus*) and the commercially cultivated genera (kelp species: *Saccharina latissima, Alaria esculenta, Laminaria digitata*) in Norwegian coastal water. These species were chosen as they are considered an important part of the future bioeconomy (Stévant et al., 2017). The activities associated with commercial macroalgae production are continuously increasing in Norway as indicated by the number of companies involved (27 in 2020 vs 10 in 2015), cultivation sites (114 in 2021 vs 83 in 2018), and harvested biomass (336 metric tons in 2020 vs 51 in 2015) (Directorate of Fisheries, 2022). The species included in this research are regarded as highly relevant for different applications, including food, livestock feed, etc., and likely to be in higher demand in the future (Makkar et al., 2016; Skjermo et al., 2014). To the best of our knowledge, this study is the first to evaluate the seasonal impacts on rumen fermentation characteristics of the brown macroalgae species: *F. vesiculosus, Fucus serratus*, and *Himanthalia elongata*. Furthermore, impacts of *F. vesiculosus*, *F. serratus*, *S. latissima,* and *Ulva lactuca* on the rumen microbiome have not been reported previously. Thus, this study aimed to test two hypotheses: A) macroalgae harvested in the spring are more rumen degradable than when harvested in the autumn. This would potentially make them more suitable as feed resources for ruminants due to higher nutritional value, and B) polyphenol-rich brown macroalgae are the most effective in reducing enteric CH_4 production, regardless of the harvesting seasons, due to their species-specific modulations of the rumen microbiome and associated rumen fermentation characteristics.

2. Materials and methods

An outline of the experimental activities and analyses is presented in Fig. 1. Prior to the harvesting of macroalgal biomass, different environmental parameters of seawater were monitored at the sampling locations.

2.1. Monitoring of environmental parameters during harvesting

Environmental parameters in the seawater (temperature, salinity, and dissolved oxygen levels) were recorded during the sampling time at seven different nearby sampling locations, using a sensor equipment STD/CTD SD204 (SAIV A/S Environmental Sensors & Systems, Bergen, Norway). The equipment was dipped about 1 m into the seawater and readings were recorded every second for at least 2 min.

2.2. Macroalgae biomass collection and processing

Twelve different macroalgae (eight brown, three red, and one green) were harvested from wild populations and processed as described previously (Roleda et al., 2019; Tayyab et al., 2016). For *Laminaria digitata*, two different parts of the thallus: blades and stipes were collected, while for other species the whole algal plants excluding the holdfast were included. In brief, about 5 kg fresh biomass for each species was collected manually from different sites within a narrow zone (N 67° 16.466′ E 014° 33.608′ to N 67° 16.550′ E 014° 34.218’) during the spring (07–09 May 2019) and autumn (01–03 October 2019) from the coastal area of Bodø, Norway (Table 1). Within 2 h of collection, the biomass for a given species collected from different sites were pooled based on similarity of environmental conditions at the harvesting sites and were transported to a laboratory (Mørkvedbukta; Nord University, Bodø) for further processing. First, the collected biomasses were washed with seawater followed by a mix of 30% seawater and 70% freshwater, and finally with freshwater to remove any possible contaminants, surface salts, and invertebrates. Then, excess water was drained and the samples were frozen at −40 °C until they were lyophilized for 72 h at −50 °C under a vacuum pressure of <0.1 mbar (Labconco, freeze dryer, Kansas City, MO, USA). Finally, the lyophilized samples were ground to a particle size of 2 mm using a cutter mill (CT Cyclotex TM 193 TM, FOSS, Hillerød, Denmark), and the homogenized material was analyzed for chemical compositions and used for *in vitro* rumen fermentation studies.

2.3. Chemical composition analyses

Dry matter (DM), organic matter (OM), ash, and protein contents of the samples were determined gravimetrically following the principles of the Association of Official Analytical Chemists (AOAC) with some modifications (Horwitz, 2010). Dry matter was estimated by drying the ground macroalgal powder at 105 °C for 24 h. Ash content was estimated by weighing the obtained residue after combustion of samples at 530 °C overnight, and OM was calculated as DM weight minus the

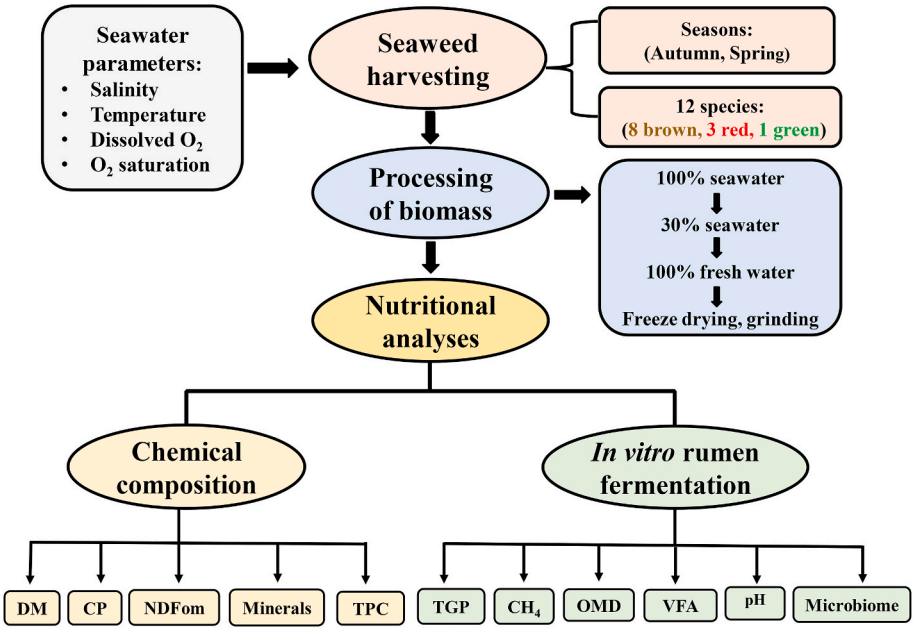

Fig. 1. A flowchart of the macroalgae experiment. Twelve different macroalgae species were harvested from the coastal area of Bodø, Norway, in two different seasons (autumn and spring). Environmental parameters of seawater at harvesting location, chemical compositions, and impacts of macroalgae on *in vitro* rumen fermentation parameters were analyzed. CH_4, Methane; CP, Crude protein; DM, Dry matter; NDFom, Ash corrected neutral detergent fiber; O_2, Oxygen; OMD, Organic matter rumen degradability; TGP, Total gas production; TPC, Total polyphenol content; VFA, Volatile fatty acids.

Table 1
List of harvested macroalgae and their phylum.

Name of macroalgae	Phylum	Harvest time (Autumn)	Harvest time (Spring)	Tidal zone[a, b]
Alaria esculenta	Brown	01 October 2019	07 May 2019	Lower intertidal to subtidal
Ascophyllum nodosum	Brown	03 October 2019	08 May 2019	Mid intertidal
Fucus serratus	Brown	03 October 2019	07 May 2019	Mid intertidal
Fucus vesiculosus	Brown	03 October 2019	08 May 2019	Mid intertidal
Himanthalia elongata	Brown	01 October 2019	09 May 2019	Lower intertidal
Laminaria digitata (blade)	Brown	02 October 2019	09 May 2019	Lower intertidal to subtidal
Laminaria digitata (stipe)	Brown	02 October 2019	09 May 2019	Lower intertidal to subtidal
Pelvetia canaliculata	Brown	03 October 2019	08 May 2019	Upper intertidal
Saccharina latissima	Brown	01 October 2019	09 May 2019	Lower intertidal to subtidal
Chondrus crispus	Red	01 October 2019	08 May 2019	Lower intertidal
Palmaria palmata	Red	01 October 2019	07 May 2019	Lower intertidal
Porphyra umbilicalis	Red	02 October 2019	09 May 2019	Lower intertidal
Ulva lactuca	Green	02 October 2019	08 May 2019	Lower intertidal

For *Laminaria digitata* two structural variants (blade and stipe) were harvested separately.

[a] Connan, S., Goulard, F., Stiger, V., Deslandes, E., Ar Gall, E., 2004. Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. DOI: https://doi.org/10.1515/BOT.2004.057

[b] Makkar, Harinder PS, Gilles Tran, Valérie Heuzé, Sylvie Giger-Reverdin, Michel Lessire, François Lebas, and Philippe Ankers. 2016. 'Seaweeds for livestock diets: a review', *Animal Feed Science and Technology*, 212: 1–17.

weight of the ash in the DM. N content was determined by the Kjeldahl method (Kjeltec™ 8400, FOSS Denmark, Hillerød, Denmark) and crude protein (CP) content was calculated using a nitrogen to protein conversion factor of 5, as previously recommended for macroalgae (Angell et al., 2016b). Neutral detergent fiber (NDF) was determined by the filter bag technique (Ankom^{200} Fiber Analyzer, NY, USA) using a neutral detergent solution, with addition of heat-stable alpha-amylase (ANKOM Technology, Macedon, NY, USA), and sodium sulfite (ANKOM Technology, Macedon, NY, USA). The residue obtained was incinerated at 550 °C for 12 h to obtain ash corrected NDF (NDFom).

The contents of individual minerals were determined after a predigestion of 150 mg of samples in a mixture of concentrated nitric acid and hydrogen peroxide (5:1, v/v) using a D Microwave digestion system (Milestone Srl, Sorisole, BG, Italy). Macrominerals (Na, K, Ca, and Mg) and trace minerals (Mn, Fe, Zn, and Cu) were determined by atomic absorption using a Microwave Plasma Atomic Emission Spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA) following the standard protocols of the Official Journal of the European Union 2009 (European Commission, 2009). A calibration curve was created by preparing sets of standard solutions with known concentrations of analytes, and the concentrations of minerals in samples were then determined from the linear regression equation.

2.4. Determination of total polyphenol contents (TPC)

The total polyphenol fraction was extracted in duplicate samples and quantified using the protocol of (Zhang et al., 2006) with some modifications. In brief, 0.5 g of ground macroalgae material was added with 10 mL of methanol-water (1:1 v/v) solution (Merck KGaA, Darmstadt, Germany), pH adjusted to ~2, and shaken (200 rpm) in an orbital shaker at room temperature for 1 h in darkness. The supernatant was recovered after centrifugation at 12000 × g_{av} for 10 min, and the residue thereafter re-extracted with 10 mL of acetone-water (7:3 v/v) solution (Merck KGaA, Darmstadt, Germany) under similar conditions as described for the methanol-water treatment. The supernatants from both extractions were pooled to make the final polyphenol extract, which was diluted 10 times in distilled water for the quantification of TPC.

For quantification of TPC in extracts, a seven-point standard curve was prepared. First, a stock standard solution (500 μg mL^{-1}) of phloroglucinol dihydrate (Acros Organics, Geel, Belgium) was prepared, and the solution was serially diluted to make standards containing 250, 125, 62.5, 31.25, 15.625, and 0 μg mL^{-1} phloroglucinol. Then, TPCs in macroalgae extracts, standard solutions, and blanks were determined in triplicates in a 96-well microplate (Thermo Fischer GmbH, Kandel, Germany) as previously described (Zhang et al., 2006) using a spectrophotometric microplate reader (absorbance at λ750 nm; BIO-RAD,

iMark™ Microplate Reader, California, USA). The mean TPC was calculated as milligram of phloroglucinol equivalents (mg PGE) per g of DM using the formula given in equation (1).

$$\text{TPC(mg PGE/g DM)} = \frac{(Mean\ TPC\ of\ sample(\mu g/mL)xSVxDF)}{DM\ weight\ of\ sample(g)x1000} X100\% \quad (1)$$

where, Mean TPC = average of the total polyphenol concentrations of triplicate samples obtained from the calibration curve, SV= Volume of solvent used for extraction, DF = Dilution factor of the original extract during the quantification assay.

2.5. In vitro ruminal gas fermentation and degradability

2.5.1. Fermentation procedure

The impact of macroalgae on rumen fermentation and gas production was simulated *in vitro* using the ANKOM^{RF} gas production system version 11.4 (Macedon, NY, USA). First, a pilot study was performed to evaluate the *in vitro* fermentation characteristics of pure macroalgae material. Thereafter, further *in vitro* fermentation studies were undertaken using macroalgae as an additive to a standard feed, maize silage (MS), in a ratio of 1:4 (w/w) giving a macroalgal inclusion rate of 20% in DM. The selection of 20% DM inclusion level was also based on previously published literature (de la Moneda et al., 2019; Maia et al., 2019). It should be noted that individual macroalgae were added to MS, and no blend of macroalgae species was used in this study.

Rumen fluid, as a source of rumen microorganisms, was obtained from two rumen-cannulated Danish Jersey heifers maintained at the Large Animal Hospital, University of Copenhagen, Denmark, following the guidelines of the Danish National Committee for the Protection of Animals used for Scientific Purposes (License nr: 2012-15-2934-00648). The donor heifers were fed a basal diet of grass silage containing 612 g/kg NDF, 72 g/kg CP, and 11 g/kg crude fat. The rumen fluids, with solids, were collected before the morning feeding in pre-warmed (39.5 °C) thermal jugs, immediately transported to the laboratory, and filtered through a double-layered cheesecloth. The filtered rumen fluid from each heifer was pooled in equal amounts and mixed with two parts of a buffer solution, containing micro-and macrominerals as well as a redox agent (Menke et al., 1979). This mixture was maintained at 39.5 °C under anaerobic conditions by continuous flushing with CO_2 gas. Ninety mL of buffered rumen fluid was dosed into a prewarmed 100 mL Duran® glass bottle that contained a feed mixture of 0.1 g macroalgae and 0.4 g MS or 0.5 g of MS or no feed (blanks), where samples had been randomly assigned to bottles. After dosing, the bottles were directly flushed with N_2 gas (to ensure anaerobic conditions and to remove any residual CO_2 present before the microbial degradation of feed) and fitted with an automatic wireless ANKOM module (ANKOM Technology, Macedon, NY, USA). The bottles were then incubated in a thermoshaker (Gerhardt Analytical Systems, Germany) at 39.5 °C with an oscillation of 40 rpm for 48 h. Duplicates of each sample type were incubated, and the fermentation was repeated twice, producing a total of four replicates per sample. To evaluate the CH_4 production, headspace gas samples were collected in gas-tight evacuated sample bags (SKC, Flex Foil PLUS) that were attached to the vent valve tube of the ANKOM module.

2.5.2. Gas recordings and rumen feed degradability determination

The pressure generated by gases in the headspace of each fermentation bottle was recorded directly to a computer connected to the ANKOM^{RF} Gas Production System. The live time was set to 60 s, pressure readings were recorded at 10-min intervals, and global release pressure was set to 0.75 psi. The cumulative pressure readings of samples from 48 h of incubation were corrected for blanks and then converted to total gas production (TGP) volumes (mL) per gram OM of feed under standard temperature and pressure conditions using the ideal gas law.

At the end of the 48 h incubation period, the fermentation bottles were transferred into an ice bath. The fluid with undegraded feed residue in the bottles was thereafter filtered through an Ankom filter bag (F57, ANKOM Technology, Macedon, NY, USA, pore size: 25 μm) and final pH of the fermentation fluid was recorded. The DM and OM contents of the undegraded feed residues retained in the filter bags were determined gravimetrically. The organic matter degradability (OMD) of the feed samples was calculated from the OM in material initially added to incubation bottles subtracted by OM in the filtered residual. The values were corrected for the increased weight of blank bags containing microbial biomass from the rumen fluid added to incubation bottles.

2.5.3. Methane measurements

Methane concentration in the gas produced over the 48 h of *in vitro* fermentation was determined by gas chromatography (GC) (Agilent 7820A GC, Agilent Technologies, Santa Clara, CA, USA). The GC equipment consisted of a HPPLOT Q column (30 m × 0.53 mm × 40 μmm) and a thermal conductivity detector that was set to 250 °C. The column flow was maintained to 5 mL/min, whereas the reference and makeup flow was adjusted to 10 mL/min. Hydrogen was used as a carrier gas. From each gas bag, a 250 μL gas sample was injected into the GC machine and run for 3 min at an isothermal oven temperature of 50 °C, and this process was performed twice for each gas sample. To calculate the CH_4 percentage in gas samples, a calibration curve made from standards containing 1%, 2.5%, 5%, 10%, 15%, and 25% of CH_4 in N_2 gas (Mikrolab A/S, Aarhus, Denmark) was used. The total volume of CH_4 produced was thereafter calculated from the CH_4 percentage multiplied by TGP.

2.5.4. Analysis of volatile fatty acids (VFAs)

For VFA analysis, samples of the liquid flow-through were collected during filtration of the fluid and undegraded feed residues after fermentation, and it was immediately mixed with 25% metaphosphoric acid solution in a 5:1 ratio (v/v) and frozen at −80 °C. Volatile fatty acids concentrations in the liquid samples were analyzed by GC (System 7890A Agilent Technologies, Santa Clara, CA, USA) as described previously (Aryal et al., 2021) and the concentrations were normalized to the VFAs produced per gram of OM fermented.

2.6. Rumen microbiome analyses using Illumina 16S rRNA amplicon sequencing

Selected samples of the liquid flow-through obtained during filtration of fluid after fermentation were used for microbiome studies. Based on *in vitro* fermentation characteristics, the samples were from blank incubations (no MS or macroalgae added to the bottles), incubations with MS alone (the basal feed), and incubations with five different macroalgae representing different rumen degradability clusters (high, medium, and low) and phylum (brown, red, and green).

2.6.1. Sample collection and DNA extraction

First, during filtration of the contents of incubation bottles through Ankom filter bags by the end of the fermentation period (see above), about 5 mL of liquid flow-through was collected in a sterile test tube and immediately frozen at −80 ^{O}C. During genomic DNA extraction, the frozen samples were thawed in ice and 1.8 mL of fluid sample was transferred into a new sterile tube and centrifuged at 10000 × g_{av} for 5 min to get cell-rich pellets (Machado et al., 2018). DNA from the cell-rich pellets was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, California, USA), and further purified using Monarch® PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA, USA) following the manufacturer's protocols. The concentration and purity of the extracted DNA were tested with NanoDrop Lite UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.2. Library preparation

The bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R

(GGACTACHVGGGTWTCTAAT), along with the Illumina Nextera overhang adapters, were used to amplify the V4 region of the bacterial 16S rRNA gene (Caporaso et al., 2011). The first PCR (PCR1) for amplification of 16S rRNA gene products was carried out in technical duplicates and pooled before adding index combination. Thermocycler conditions were 95 ^{O}C for 5 min, 35 cycles of 95 ^{O}C for 30 s, 55 ^{O}C for 30 s, 72 ^{O}C for 1 min, and the final elongation at 72 ^{O}C for 5 min (SimpliAmp Thermal Cycler, Applied Biosystems, California, USA). Each PCR reaction of 25 μl consisted of 5xPCRBIO HiFi Buffer (5 μl) (PCRBiosystems, London, UK), 10 ng of DNA template, 0.5 unit of PCRBIO HiFi Polymerase (PCRBiosystems, London, UK), 0.2 mM of forward and reverse primers, and 100 ng bovine serum. After PCR1, a second PCR (PCR2) was used to add unique index combinations (i7and i5) and adaptors. For PCR2, Thermocycler conditions were 95 ^{O}C for 5 min, 13 cycles of 95 ^{O}C for 30 s, 58 ^{O}C for 30 s, 68 ^{O}C for 1 min, and the final elongation at 68 ^{O}C for 10 min. Subsequently, the amplicon product was cleaned using HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, USA), according to the manufacturer's instructions. Finally, amplicons were pooled in equimolar concentration, and sequencing was carried out using the Illumina MiSeq platform. All the sequence files were deposited in the NCBI Sequence Read Archive (SRA) under the accession number: PRJNA780171.

2.6.3. Bioinformatics of sequencing data

The DNA reads obtained from the Illumina MiSeq run were analyzed using QIIME2 (Bolyen et al., 2019) mainly using the dada2 plugin (Callahan et al., 2016). In brief, paired-end reads were denoised, joined, dereplicated, forward and reverse primers trimmed, and finally filtered for chimeras using the 'dada2 denoise-paired' command. Following this, taxonomy to amplicon sequence variants (ASVs) was assigned via 'feature-classifier classify-consensus-vsearch' using the SILVA 132 database (Quast et al., 2012). To perform data analysis and data visualization, the ASV table and the taxonomy files were imported to the R version 4.0.3 (R Core Team, 2021). Diversity-based analysis was done using the vegan package ver. 2.5–7 (Oksanen et al., 2013) and the phyloseq package ver. 1.34 (McMurdie and Holmes, 2013).

2.7. Calculation and statistical analyses

All statistical analyses were performed using the R Foundation for Statistical Computing Platform, version 4.1.1 (R Core Team, 2021). Homogeneity of variance was evaluated by visual inspection of residual plots, and normality of residuals was tested by quantile-quantile plots. The environmental parameter data from two seasons were analyzed by unpaired *t*-test. Data for the chemical composition (ash, crude protein, NDFom, TPC, and minerals) of macroalgae, and *in vitro* rumen degradability characteristics (TGP, OMD, pH, VFA, and CH_4) were analyzed by two-way ANOVA, where fixed effects of season and species, and their interactions were used. The patterns of development of TGP during *in vitro* fermentation (broken down each hour of fermentation) were analyzed as repeated measures using a mixed effect model. The model included fixed effects of seasons, macroalgae species, incubation hours, and their interactions, while fermentation runs, and replicate numbers were used as random effects. For this, different correlation structures between measurements and heterogeneous variances were tested and the structure yielding the best fit (autoregressive first order) was chosen. In addition, the TGP data from each hour of fermentation was used for hierarchical clustering to generate a heatmap, where TGP values were center-scaled (z-transformation) across fermentation hours and the Euclidean distance matrix was generated. Then the "average" algorithm was conducted for hierarchical agglomerative clustering using ComplexHeatmap (Gu et al., 2016) and dendextend (Galili, 2015) R packages. In the end, a Spearman's correlation matrix was generated to evaluate the correlation among chemical composition, *in vitro* rumen fermentation characteristics, and rumen microbial compositions using the Corrplot package in R. Differences in the least square means (LS means) were compared by Tukey's multiple comparison test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Seawater parameters during the sampling of macroalgae

The levels of salinity and dissolved oxygen were found to be similar across both harvesting seasons. The oxygen saturation levels were significantly higher in the spring as compared to those recorded in the autumn ($P < 0.0001$), whereas the water temperature levels were higher in the autumn compared to spring ($P < 0.0001$) (Suppl. Fig. S1).

3.2. Chemical composition of macroalgae

3.2.1. Ash and minerals

Brown ($P = 0.00029$) and green ($P = 0.023$) species had higher ash contents than the red species (Table 2). The ash contents were higher in the spring than in the autumn for all species ($P < 0.05$), except for opposite trends in *F. serratus*, *L. digitata* (stipe), and *C. crispus* ($P < 0.0001$, for all three). The largest seasonal differences were observed in the brown macroalgae, *S. latissima* ($P < 0.0001$) and *L. digitata* (blade) ($P < 0.0001$), and the red macroalga, *P. palmata* ($P < 0.0001$) with ~96%, 54.5%, and ~74%, respectively, higher ash concentrations in the spring compared to the autumn.

The contents of macrominerals were generally highest in brown species followed by green and red species. The most abundant macrominerals were either K (up to 9.27% DM, e.g., *L. digitata*, *S. latissima*, *H. elongata, P. palmata*) or Na (up to 5.9% DM, e.g., *A. nodosum., F. vesiculosus, P. canaliculata, C. crispus*) (Table 3), while Fe (28–223 mg/kg DM) was the most dominant micromineral in all macroalgae species (Table 4).

The effect of season on the studied mineral elements of macroalgae was species-specific. For instance, the level of K was found higher in spring ($P < 0.0001$) except for *F.serratus, P. canaliculata,* and *P. umbilicalis*, which had higher levels in autumn ($P < 0.0001$). Similarly, Na was present in greater concentration in spring for *A. esculenta, H. elongata, F. vesiculosus, S. latissima, P. umbilicalis* (~2 fold), and *U. lactuca* (3.5 fold) as compared to the autumn ($P < 0.05$), while the opposite was the case for *C. crispus* ($P = 0.025$). The largest seasonal variation in Ca content was noted for red species, *P. umbilicalis,* and *C. crispus*, with ~5 and ~2 fold higher contents, respectively, in autumn as compared to the spring ($P < 0.05$). The Mg content was 2.7 fold higher in autumn than spring for green macroalga, *U. lactuca*, whereas it was highest in spring or unaffected by season for other species. Similar to the macrominerals, the concentration of microminerals in macroalgae was also differentially affected by the harvesting season. Two brown species: *A. nodosum* and *P. canaliculata* had higher levels of Fe, Zn, and Mn in the spring than in the autumn ($P < 0.0001$, for both), while the opposite trend was evident for another brown macroalga, *H. elongata* ($P < 0.0001$), and green macroalga, *U. lactuca* ($P < 0.0001$).

3.2.2. Crude protein

Green ($P < 0.0001$) and red ($P < 0.0001$) macroalgae had similar CP contents, which were > 2-fold higher than the levels found in brown species. Crude protein concentrations were higher in the spring ($P < 0.0001$) than autumn for all studied species ($P < 0.0001$) (Table 2). The seasonal differences were most pronounced for the brown macroalgae belonging to the Fucaceae family: *F. vesiculosus*, *F. serratus*, *A. nodosum,* and *P. canaliculata* with 86%, 74%, 73%, and 66%, respectively, higher CP levels in the spring compared to autumn (interaction of season and species: $P < 0.0001$). The green macroalga, *U. lactuca*, and red macroalgae, *C. crispus, P. palmata* and *P. umbilicalis*, were the richest (13–20% of DM) in CP while the brown macroalgae *A. nodosum* and *F. vesiculosus* had the lowest (<8% of DM for both) contents.

Table 2
Chemical composition of macroalgae harvested in autumn and spring seasons.

Species	Ash% in DM		CP (g/kg DM)		NDFom (g/kg DM)		TPC (mg PGE/gDM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	17.2 ± 0.09^{f}	$21.7 \pm 0.05^{g*}$	104.9 ± 0.64^{c}	$136.3 \pm 0.1^{c*}$	471.5 ± 10.75^{d}	487.9 ± 1.91^{de}	33.2 ± 1.6^{d}	22.3 ± 1.75^{de}
A. nodosum	20.9 ± 0.32^{d}	$24.3 \pm 0.34^{f*}$	41.1 ± 0.77^{h}	$71.3 \pm 0.38^{g*}$	579.2 ± 3.96^{a}	$537.5 \pm 6.47^{c*}$	111.9 ± 7.69^{b}	104.9 ± 2.2^{b}
F. serratus	25.2 ± 0.05^{c}	$19.5 \pm 0.08^{i*}$	60.6 ± 0.13^{ef}	$105.0 \pm 0.0^{de*}$	458.0 ± 5.02^{d}	$527.1 \pm 8.56^{c*}$	84.8 ± 5.08^{c}	94.5 ± 3.92^{b}
F. vesiculosus	18.8 ± 0.97^{e}	$24.4 \pm 0.23^{f*}$	44.0 ± 0.11^{gh}	$82.0 \pm 0.32^{f*}$	370.8 ± 5.83^{f}	$462.0 \pm 2.37^{e*}$	178.2 ± 0.99^{a}	$133.1 \pm 2.79^{a*}$
H. elongata	32.0 ± 0.06^{b}	$39.6 \pm 0.06^{a*}$	56.4 ± 0.53^{f}	$85.2 \pm 0.21^{f*}$	391.3 ± 9.63^{ef}	393.2 ± 8.07^{f}	38.1 ± 1.18^{d}	26.9 ± 0.45^{d}
L. digitata (blade)	19.6 ± 0.06^{e}	$30.3 \pm 0.04^{d*}$	65.7 ± 0.03^{de}	$98.7 \pm 0.48^{e*}$	474.3 ± 6.73^{d}	490.4 ± 2.65^{de}	7.1 ± 1.82^{ef}	6.6 ± 2.02^{f}
L. digitata (stipe)	33.7 ± 0.04^{a}	$32.2 \pm 0.02^{c*}$	57.0 ± 0.32^{f}	$70.4 \pm 0.08^{g*}$	546.9 ± 2.45^{b}	$664.1 \pm 1.2^{a*}$	6.5 ± 1.00^{ef}	6.9 ± 1.03^{f}
P. canaliculata	19.4 ± 0.13^{e}	$20.8 \pm 0.12^{h*}$	52.6 ± 0.05^{fg}	$87.3 \pm 0.16^{f*}$	507.9 ± 8.65^{c}	519.1 ± 3.07^{cd}	92.9 ± 2.7^{c}	$75.6 \pm 3.14^{c*}$
S. latissima	16.9 ± 0.19^{f}	$33.1 \pm 0.09^{b*}$	73.5 ± 0.42^{d}	$112.8 \pm 0.24^{d*}$	414.5 ± 2.02^{e}	517.4 ± 9.24^{cd}	16.5 ± 0.63^{e}	11.6 ± 0.43^{ef}
C. crispus	19.2 ± 0.12^{e}	$15.6 \pm 0.05^{j*}$	143.2 ± 0.5^{b}	$195.8 \pm 8.23^{b*}$	591.6 ± 5.12^{a}	$617.6 \pm 13.1^{b*}$	8.3 ± 1.45^{ef}	10.5 ± 2.26^{ef}
P. palmata	13.8 ± 0.03^{g}	$23.9 \pm 0.11^{f*}$	136.6 ± 0.05^{b}	$195.2 \pm 1.21^{b*}$	$363.1 \pm 3.7^{*f}$	$321.6 \pm 0.97^{g*}$	6.5 ± 1.17^{ef}	8.2 ± 0.93^{f}
P. umbilicalis	10.5 ± 0.04^{h}	$13.5 \pm 0.16^{k*}$	143.8 ± 0.51^{ab}	$194.8 \pm 0.47^{b*}$	$599.3 \pm 3.93^{*a}$	$543.8 \pm 3.74^{c*}$	7.6 ± 0.75^{ef}	10.2 ± 1.44^{f}
U. lactuca	25.1 ± 0.05^{c}	$26.0 \pm 0.06^{e*}$	152.0 ± 0.19^{a}	$204.6 \pm 0.72^{a*}$	$271.7 \pm 5.1^{*g}$	$159.9 \pm 3.14^{h*}$	2.6 ± 0.06^{f}	4.1 ± 0.65^{f}
Maize silage	4.3 ± 0.01		80.2 ± 0.57		402.1 ± 3.4		9.4 ± 0.04	

Results are presented as mean ± standard errors of the mean of duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring; CP, Crude protein; NDFom, Ash corrected neutral detergent fiber; TPC, Total polyphenol content; PGE, Phloroglucinol equivalents. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

Table 3
Composition of macro minerals in macroalgae.

Species	K (% of DM)		Na (% of DM)		Ca (% of DM)		Mg (% of DM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	4.10 ± 0.05^{bc}	$5.95 \pm 0.01^{d*}$	1.77 ± 0.02^{efg}	1.94 ± 0.05^{e}	1.01 ± 0.01^{cd}	0.93 ± 0.05^{bc}	0.54 ± 0.01^{fg}	$0.68 \pm 0.03^{e*}$
A. nodosum	1.72 ± 0.04^{g}	$2.21 \pm 0.05^{g*}$	3.16 ± 0.05^{bc}	3.62 ± 0.02^{c}	1.00 ± 0.02^{cd}	1.03 ± 0.01^{bc}	0.79 ± 0.02^{cd}	0.84 ± 0.01^{bc}
F. serratus	4.12 ± 0.07^{b}	$3.33 \pm 0.02^{e*}$	3.23 ± 0.05^{b}	2.80 ± 0.02^{cde}	1.10 ± 0.02^{c}	1.01 ± 0.00^{bc}	0.75 ± 0.01^{cd}	0.69 ± 0.01^{de}
F. vesiculosus	2.26 ± 0.00^{e}	$2.81 \pm 0.08^{f*}$	2.34 ± 0.01^{cdef}	$3.08 \pm 0.08^{cd*}$	0.89 ± 0.00^{cd}	$1.09 \pm 0.01^{bc*}$	0.63 ± 0.00^{ef}	$0.73 \pm 0.02^{cde*}$
H. elongata	4.22 ± 0.01^{b}	$7.56 \pm 0.01^{b*}$	4.72 ± 0.10^{a}	$5.90 \pm 0.00^{a*}$	2.16 ± 0.02^{a}	$1.12 \pm 0.01^{b*}$	0.98 ± 0.00^{b}	$1.07 \pm 0.01^{a*}$
L. digitata (blade)	3.85 ± 0.02^{b}	$7.57 \pm 0.10^{b*}$	3.06 ± 0.48^{bc}	3.14 ± 0.56^{cd}	1.09 ± 0.16^{c}	1.11 ± 0.11^{bc}	0.68 ± 0.06^{de}	0.69 ± 0.08^{de}
L. digitata (stipe)	9.27 ± 0.11^{a}	$8.41 \pm 0.00^{a*}$	3.17 ± 0.03^{bc}	3.33 ± 0.00^{c}	1.35 ± 0.01^{b}	$1.47 \pm 0.03^{a*}$	0.74 ± 0.03^{cd}	0.80 ± 0.00^{cd}
P. canaliculata	1.98 ± 0.00^{f}	$1.65 \pm 0.03^{h*}$	2.61 ± 0.00^{bcde}	2.94 ± 0.05^{cd}	1.05 ± 0.01^{cd}	$0.90 \pm 0.00^{c*}$	0.82 ± 0.00^{c}	0.81 ± 0.01^{c}
S. latissima	3.99 ± 0.04^{bc}	$6.99 \pm 0.07^{c*}$	2.03 ± 0.02^{defg}	$2.94 \pm 0.01^{cd*}$	0.84 ± 0.01^{d}	0.91 ± 0.00^{bc}	0.50 ± 0.00^{gh}	$0.73 \pm 0.00^{cde*}$
C. crispus	1.67 ± 0.02^{g}	1.53 ± 0.01^{h}	2.81 ± 0.01^{bcd}	$2.35 \pm 0.01^{de*}$	1.07 ± 0.00^{c}	$0.51 \pm 0.00^{d*}$	0.68 ± 0.00^{de}	0.68 ± 0.00^{e}
P. palmata	2.18 ± 0.05^{ef}	$2.59 \pm 0.00^{f*}$	0.19 ± 0.00^{k}	0.65 ± 0.00^{f}	0.20 ± 0.00^{f}	0.15 ± 0.00^{e}	0.10 ± 0.00^{i}	0.15 ± 0.00^{g}
P. umbilicalis	2.02 ± 0.02^{ef}	$1.28 \pm 0.01^{i*}$	1.55 ± 0.01^{h}	$3.01 \pm 0.28^{cd*}$	1.07 ± 0.01^{c}	$0.21 \pm 0.00^{e*}$	0.39 ± 0.00^{h}	$0.49 \pm 0.00^{f*}$
U. lactuca	2.74 ± 0.04^{d}	2.67 ± 0.03^{f}	1.30 ± 0.01^{g}	$4.62 \pm 0.00^{b*}$	0.52 ± 0.00^{e}	0.58 ± 0.00^{d}	2.62 ± 0.01^{a}	$0.96 \pm 0.01^{b*}$
Maize silage	1.28 ± 0.08		0.01 ± 0.00		0.18 ± 0.00		0.10 ± 0.01	

Results are presented as mean ± standard error of the mean for duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

Table 4
Composition of microminerals in macroalgae.

Species	Fe (mg/kg DM)		Zn (mg/kg DM)		Mn (mg/kg DM)		Cu (mg/kg DM)	
	Aut	Spr	Aut	Spr	Aut	Spr	Aut	Spr
A. esculenta	57.6 ± 0.31^{f}	$114.8 \pm 9.11^{c*}$	42.1 ± 0.03^{c}	$33.3 \pm 2.57^{cde*}$	3.3 ± 0.13^{h}	$8.4 \pm 0.26^{fg*}$	2.6 ± 0.00^{cd}	2.2 ± 0.13^{ef}
A. nodosum	28.3 ± 0.60^{g}	$51.2 \pm 2.20^{hi*}$	17.5 ± 1.30^{gh}	$26.7 \pm 0.03^{f*}$	7.8 ± 0.25^{f}	$13.3 \pm 0.25^{e*}$	1.6 ± 0.00^{de}	1.9 ± 0.00^{ef}
F. serratus	86.5 ± 0.86^{de}	84.1 ± 0.90^{de}	63.5 ± 0.05^{b}	$85.0 \pm 0.53^{a*}$	65.4 ± 0.58^{a}	$59.7 \pm 0.02^{a*}$	6.8 ± 0.14^{a}	$9.81 \pm 0.04^{a*}$
F. vesiculosus	138.9 ± 0.89^{b}	$97.7 \pm 0.68^{cd*}$	18.3 ± 0.01^{g}	$44.5 \pm 0.01^{b*}$	44.1 ± 0.36^{b}	$46.7 \pm 0.90^{b*}$	2.1 ± 0.00^{de}	$4.1 \pm 0.65^{cd*}$
H. elongata	109.9 ± 1.98^{c}	$44.6 \pm 0.50^{i*}$	30.2 ± 1.42^{de}	$25.9 \pm 0.08^{f*}$	39.0 ± 0.54^{c}	$34.6 \pm 0.02^{c*}$	4.6 ± 0.11^{b}	$2.6 \pm 0.01^{ef*}$
L. digitata (blade)	79.3 ± 0.37^{e}	$45.3 \pm 0.64^{i*}$	31.5 ± 0.09^{d}	$34.5 \pm 0.02^{c*}$	3.3 ± 0.14^{h}	4.0 ± 0.00^{i}	1.6 ± 0.00^{de}	1.6 ± 0.00^{f}
L. digitata (stipe)	52.9 ± 0.80^{f}	$59.5 \pm 1.74^{gh*}$	13.3 ± 0.03^{h}	$29.2 \pm 0.14^{ef*}$	1.9 ± 0.00^{h}	2.1 ± 0.01^{j}	2.3 ± 0.14^{cde}	2.1 ± 0.28^{ef}
P. canaliculata	80.3 ± 2.28^{e}	$223.3 \pm 9.59^{a*}$	26.3 ± 0.07^{ef}	$33.6 \pm 0.03^{cd*}$	8.3 ± 0.11^{f}	$9.6 \pm 0.01^{f*}$	3.6 ± 0.12^{bc}	3.1 ± 0.00^{de}
S. latissima	98.3 ± 0.02^{cd}	$80.3 \pm 0.27^{ef*}$	15.6 ± 0.04^{gh}	$33.2 \pm 0.11^{cde*}$	3.0 ± 0.12^{h}	$6.7 \pm 0.02^{h*}$	1.0 ± 0.00^{e}	2.6 ± 0.01^{ef}
C. crispus	156.7 ± 0.75^{b}	162.8 ± 1.87^{b}	73.4 ± 0.20^{a}	$85.6 \pm 0.04^{a*}$	7.3 ± 0.02^{g}	7.0 ± 0.26^{gh}	6.7 ± 0.90^{a}	$4.4 \pm 0.26^{cd*}$
P. palmata	144.1 ± 0.13^{b}	$96.4 \pm 0.10^{cd*}$	22.9 ± 0.04^{f}	$34.2 \pm 1.27^{c*}$	5.6 ± 0.01^{f}	$9.1 \pm 0.00^{f*}$	4.8 ± 0.01^{b}	$4.2 \pm 0.13^{cd*}$
P. umbilicalis	62.1 ± 2.03^{f}	$70.2 \pm 7.53^{fg*}$	44.4 ± 1.08^{c}	$29.4 \pm 1.23^{def*}$	16.4 ± 0.31^{d}	$12.5 \pm 0.02^{e*}$	6.7 ± 0.10^{a}	$4.7 \pm 0.12^{c*}$
U. lactuca	214.8 ± 1.19^{a}	$189.7 \pm 5.05^{ab*}$	BDL	$11.7 \pm 1.28^{g*}$	10.2 ± 0.12^{e}	$15.2 \pm 0.36^{d*}$	4.7 ± 0.13^{b}	$6.4 \pm 0.12^{b*}$
Maize silage	154.6 ± 0.19		10.7 ± 0.06		25.7 ± 0.15		7.35 ± 0.18	

Results are presented as mean ± standard error of the mean for duplicate analyses. DM, Dry matter; Aut, Autumn; Spr, Spring; BDL, Below the detectable level. Species not sharing the same letters in the superscripts within a column are significantly different. Species with * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season.

3.2.3. Ash corrected neutral detergent fiber

The NDFom content was higher in brown and red species compared to green species ($P < 0.0001$ for both) (Table 2). However, a strong interaction between species and harvesting season was observed ($P < 0.0001$), since some species (*C. crispus*, *F. serratus, F. vesiculosus*, *S. latissima*) had higher NDFom contents in the spring ($P < 0.0062$, for all), whereas others (*A. nodosum, L. digitata* (stipe), *P. palmata, P. umbilicalis* and *U. lactuca*) had highest contents in the autumn ($P < 0.0001$, for all). The red macroalga, *C. crispus* (in spring) had the highest content of NDFom (62% of DM), while the lowest content (16% of DM) was found in green macroalga *U. lactuca* (in autumn).

3.2.4. Total polyphenol contents

As expected, brown macroalgae had higher TPC (10–20 times) as compared to red and green species, except for a low level in *L. digitata*, which was similar to those seen in the red species (season and species interaction: $P = 0.0002$; Table 2). The highest TPC contents were found in four species from the Fucaceae family: *A. nodosum, F. vesiculous, F. serratus,* and *P. canaliculata* (>75 mg PGE per gram DM). Seasonal variations in TPC were observed in two brown species, *F. vesiculosus* ($P < 0.0001$) and *P. canaliculata* ($P < 0.0001$), with higher values in the autumn. A similar trend was also observed for *H. elongata* ($P = 0.078$) and *A. esculenta* ($P = 0.083$), with up to 49% higher TPC levels in the autumn than in the spring.

3.3. In vitro gas production by pure (sole) macroalgae

The *in vitro* study, with pure macroalgal species fermented alone as a substrate, showed that macroalgae produce remarkably less total gas than the standard ruminant feed, maize silage (MS), regardless of species and harvesting time (Suppl. Fig. S2). *S. latissima* and *A. esculenta* had the highest TGP among the tested species during the 48 h of fermentation, however, such productions were ~40 and 47%, respectively, lower than the TGP of MS.

3.4. Effects of addition of macroalgae to maize silage on in vitro rumen fermentation characteristics

3.4.1. Organic matter degradability

When individual macroalgae were co-fermented with MS, no impact of the macroalgae harvesting season was observed on OMD of the feed mixture (individual macroalgae + MS), except for *A. esculenta* ($P = 0.0001$) and *A. nodosum* ($P = 0.017$) harvested in the spring that resulted in higher OMD than from the autumn (Table 5). However, when compared to the MS fermented alone, using the following four brown macroalgae as additives suppressed total OMD by 12–25%, regardless of harvesting season: *A. nodosum* ($P < 0.0001$), *F. vesiculosus* ($P < 0.0001$), *F. serratus* ($P < 0.0001$), *P. canaliculata* ($P < 0.0001$), and the red macroalga *C. crispus* ($P = 0.0037$) (Suppl. Fig. S3). In contrast, *P. umbilicalis* ($P = 0.023$) and *A. esculenta* ($P = 0.0001$) reduced total OMD by ~8–12%, but only when harvested in the autumn. Other brown species, *L. digitata*, *S. latissima,* and *H. elongata,* the red species, *P. palmata,* and the green species, *U. lactuca*, caused no or only marginal changes in total OMD.

3.4.2. Volatile fatty acid profiles

The addition of spring harvested *A. esculenta* ($P = 0.002$), *A. nodosum* ($P = 0.03$), and *F. vesiculosus* ($P = 0.003$), resulted in a higher total concentration of VFAs in the post-fermentation rumen fluid by the end of the 48-h fermentation period, as compared to the same macroalgae harvested in the autumn (Table 6). Such differences in total VFA were in agreement with differences in OMD except for *F. vesiculosus*. This was

Table 5
Impacts of addition of macroalgae (20% of total DM) to maize silage (1:4 ratio ~20% macroalgae DM in the mix) *in vitro* rumen fermentation characteristics (48 h).

Species	TGP (ml/gOM)		pH		CH_4 (% of TGP)		CH_4 (ml/gOM)		OMD (%)	
	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring
AE	150.3 ± 3.09^{bcde}	160.2 ± 2.97^{abcd}	6.9 ± 0.04	6.9 ± 0.02	7.4 ± 0.56^{ab}	8.3 ± 0.69^{a}	11.1 ± 0.85^{abc}	13.3 ± 1.41^{a}	64.1 ± 1.57$^{de\#}$	70.4 ± 0.53^{abc*}
AN	133.2 ± 1.71$^{ef\#}$	140.8 ± 3.13$^{de\#}$	7.0 ± 0.06	7.0 ± 0.04	5.7 ± 0.06^{ab}	6.8 ± 1.30^{ab}	7.5 ± 0.02^{bc}	9.5 ± 1.57^{ab*}	54.8 ± 0.89$^{g\#}$	59.3 ± 0.86$^{e*\#}$
FS	142.8 ± 4.11^{cde}	139.4 ± 2.99$^{de\#}$	6.9 ± 0.03	6.9 ± 0.03	6.0 ± 0.17^{ab}	7.0 ± 0.05^{ab}	8.5 ± 0.60^{abc}	9.8 ± 0.38^{ab}	60.6 ± 1.22$^{ef\#}$	61.5 ± 2.23$^{de\#}$
FV	113.2 ± 5.91f$^{\#}$	128.3 ± 2.35$^{e*\#}$	7.0 ± 0.04	7.0 ± 0.04	4.6 ± 0.80$^{b\#}$	7.0 ± 0.72^{ab*}	5.4 ± 1.09$^{c\#}$	8.9 ± 1.07^{ab*}	55.7 ± 2.00$^{fg\#}$	56.9 ± 0.82$^{e\#}$
HE	166.3 ± 5.38^{abc}	158.7 ± 3.00^{abcd}	6.9 ± 0.02	7.0 ± 0.03	8.2 ± 0.09^{a}	7.4 ± 0.18^{ab}	13.6 ± 0.18^{a}	11.3 ± 0.47^{ab}	67.8 ± 1.46^{bcd}	70.1 ± 0.24^{abc}
LD	173.6 ± 5.42^{ab}	163.2 ± 4.65^{abcd}	6.9 ± 0.04	6.9 ± 0.03	6.4 ± 0.0^{ab}	7.9 ± 0.25^{ab}	11.3 ± 0.52^{a}	12.8 ± 0.92^{ab}	72.8 ± 1.77^{ab}	70.9 ± 0.25^{ab}
LS	173.8 ± 7.86^{ab}	175.1 ± 3.50^{a}	6.9 ± 0.04	7.0 ± 0.04	7.6 ± 0.90^{ab}	7.9 ± 0.29^{a}	13.3 ± 0.96^{ab}	13.8 ± 0.03^{a}	71.6 ± 0.19^{ab}	73.7 ± 2.03^{a}
PC	142.3 ± 4.96^{de}	152.8 ± 4.03^{abcd}	7.0 ± 0.04	7.0 ± 0.05	7.2 ± 1.02^{ab}	7.8 ± 1.25^{ab}	10.4 ± 2.03^{abc}	12.0 ± 2.43^{ab}	60.1 ± 1.15$^{\#}$	61.7 ± 1.74$^{de\#}$
SL	163.4 ± 6.14^{abcd}	166.7 ± 4.59^{abc}	6.9 ± 0.02	6.9 ± 0.04	8.1 ± 0.22^{ab}	7.9 ± 0.75^{ab}	13.2 ± 0.11^{ab}	13.1 ± 1.24^{a}	70.1 ± 0.46^{abc}	70.6 ± 0.51^{abc}
CC	142.7 ± 3.3^{cde}	146.8 ± 1.01^{bcde}	7.0 ± 0.03	7.0 ± 0.06	8.5 ± 0.44^{a}	4.3 ± 0.35$^{b*\#}$	12.5 ± 1.25^{ab}	6.3 ± 0.52$^{b\#}$	64.2 ± 0.61$^{de*\#}$	65.5 ± 1.38$^{cd\#}$
PP	183.0 ± 7.25^{a}	169.3 ± 3.05^{ab*}	7.0 ± 0.06	7.0 ± 0.04	7.5 ± 1.29^{ab}	7.4 ± 0.83^{ab}	13.6 ± 1.71^{a}	12.6 ± 1.50^{ab}	73.5 ± 2.03^{a}	73.5 ± 1.42^{a}
PU	153.9 ± 8.61^{bcde}	143.3 ± 11.30^{cde}	7.0 ± 0.03	7.0 ± 0.01	6.7 ± 0.0^{ab}	5.6 ± 0.37^{ab}	10.3 ± 0.08^{abc}	8.2 ± 0.16^{ab}	66.4 ± 2.19$^{cd\#}$	68.5 ± 2.09^{bc}
UL	156.3 ± 2.40^{abcd}	155.9 ± 3.92^{abc}	6.9 ± 0.03	7.0 ± 0.03	5.7 ± 0.49^{ab}	6.3 ± 1.22^{ab}	8.9 ± 0.62^{abc}	9.8 ± 1.96^{ab}	72.6 ± 1.11^{ab}	72.1 ± 1.30^{ab}
MS	167.7 ± 5.00		6.9 ± 0.06		8.7 ± 0.49		14.5 ± 0.75		72.8 ± 2.39	

Results are presented as mean values ± standard error of the mean (n = 4 for OMD, TGP, and pH, and n = 2 for CH_4). AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus serratus*; FV, *Fucus vesiculosus*; HE, *Himanthalia elongata*; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculata*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*; MS, Maize silage. DM, Dry matter;/gOM, Per gram organic matter; TGP, Total gas production; OMD, Organic matter degradability. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season. Species with the # sign in the superscript are significantly different from maize silage (MS) fermented alone.

Table 6
Effects of macroalgae inclusion on concentrations of volatile fatty acids at 48 h of *in vitro* rumen fermentation.

Species	Total VFA		Acetic acid		Propionic acid		Butyric acid		Total Minor VFAs	
	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring
A. esculenta	33.4 ± 2.83^{abcd}	37.8 ± 0.97^{ab*}	20.4 ± 2.23^{abc}	24.2 ± 0.80^{a*}	9.7 ± 0.82^{ab}	8.3 ± 0.81*	2.6 ± 0.29$^{cde\#}$	4.0 ± 0.82*	0.70 ± 0.07^{cd}	1.35 ± 0.15^{abc*}
A. nodosum	28.1 ± 3.16$^{de\#}$	32.3 ± 0.84^{abc*}	18.2 ± 1.96^{cd}	21.1 ± 0.43^{abc*}	7.0 ± 1.73^{d}	7.5 ± 1.11	2.5 ± 0.64$^{de\#}$	3.1 ± 0.77	0.38 ± 0.06^{de}	0.56 ± 0.08^{ef}
F. serratus	30.8 ± 2.84^{cd}	31.2 ± 0.86^{bc}	19.4 ± 1.84^{bc}	19.5 ± 0.07^{bc}	8.0 ± 1.49^{abcd}	7.9 ± 0.49	2.9 ± 0.68$^{bcde\#}$	3.3 ± 1.26	0.53 ± 0.19^{de}	0.57 ± 0.01^{ef}
F. vesiculosus	23.1 ± 2.51$^{e\#}$	29.0 ± 0.19$^{c*\#}$	13.8 ± 1.45$^{d\#}$	18.6 ± 0.53^{c*}	7.1 ± 1.87^{d}	7.5 ± 0.82	2.1 ± 0.82$^{e\#}$	2.9 ± 1.17$^{*\#}$	0.17 ± 0.02^{e}	0.37 ± 0.02^{f}
H. elongata	36.4 ± 0.56^{abc}	34.3 ± 1.62^{abc}	23.7 ± 0.33^{ab}	22.2 ± 1.15^{abc}	8.0 ± 0.95^{abcd}	7.3 ± 1.04	3.7 ± 0.76^{abcd}	3.8 ± 0.67	0.98 ± 0.04^{bc}	1.04 ± 0.10^{cd}
L. digitata (blade)	39.4 ± 0.65^{a}	36.9 ± 1.48^{ab}	24.3 ± 0.54^{a}	22.6 ± 1.2^{abc}	9.8 ± 0.58^{a}	8.9 ± 0.85	4.0 ± 0.5^{ab}	4.1 ± 0.65	1.29 ± 0.04^{ab}	1.31 ± 0.08^{abc}
L. digitata (stipe)	36.8 ± 2.18^{abc}	37.1 ± 2.57^{ab}	23.3 ± 1.73^{abcd}	23.9 ± 1.92^{ab}	8.2 ± 0.99^{abcd}	8.1 ± 1.24	3.8 ± 0.49^{abc}	4.0 ± 0.73	1.35 ± 0.05^{ab}	1.20 ± 0.13^{abcd}
P. canaliculata	31.4 ± 0.15^{bcd}	34.2 ± 3.39^{abc}	19.9 ± 0.04^{abc}	21.6 ± 2.44^{abc}	7.7 ± 0.81^{bcd}	8.0 ± 1.51	3.3 ± 1.04^{abcde}	3.8 ± 0.73	0.54 ± 0.05^{de}	0.84 ± 0.16^{de*}
S. latissima	37.7 ± 1.81^{ab}	38.3 ± 0.30^{a}	23.2 ± 1.47^{ab}	24.2 ± 0.35^{a}	9.7 ± 0.56^{ab}	9.0 ± 0.46	3.7 ± 0.26^{abcd}	3.8 ± 0.53	1.19 ± 0.03^{ab}	1.32 ± 0.04^{abc}
C. crispus	32.9 ± 0.15^{abcde}	32.0 ± 1.56^{abc}	20.9 ± 0.42^{abc}	20.1 ± 1.15^{abc}	7.4 ± 0.8^{cd}	7.1 ± 0.91	3.5 ± 0.59^{abcd}	3.7 ± 0.61	1.11 ± 0.06^{abc}	1.13 ± 0.12^{bcd}
P. palmata	37.9 ± 1.84^{ab}	36.4 ± 1.93^{ab}	22.8 ± 1.54^{ab}	22.1 ± 1.76^{abc}	9.3 ± 0.7^{abc}	8.6 ± 0.5	4.3 ± 0.45^{a}	4.1 ± 0.41	1.44 ± 0.06^{a}	1.57 ± 0.08^{a}
P. umbilicalis	37.3 ± 1.85^{ab}	34.0 ± 1.20^{abc}	23.6 ± 0.84^{ab}	21.1 ± 0.46^{abc}	8.2 ± 1.42^{abcd}	7.5 ± 0.24	4.1 ± 0.3^{ab}	4.1 ± 0.92	1.40 ± 0.12^{a}	1.32 ± 0.04^{abc}
U. lactuca	34.9 ± 0.78^{abc}	35.6 ± 0.80^{abc}	21.8 ± 0.81^{abc}	22.2 ± 0.83^{abc}	7.9 ± 0.77^{abcd}	7.9 ± 0.94	4.0 ± 0.83^{ab}	4.0 ± 0.83	1.26 ± 0.04^{ab}	1.50 ± 0.15^{ab*}
Maize silage	37.0 ± 3.07		22.6 ± 2.18		8.7 ± 1.56		4.4 ± 0.77		1.32 ± 0.09	

The amounts of VFAs (Volatile fatty acids) are presented in mmol/L produced per gram of organic matter. At the end of fermentation, the fermentation medium was filtered, and fluid was mixed with 25% metaphosphoric acid at a 1:5 ratio (v/v) and VFAs were analyzed by gas chromatography. Results are presented as mean ± standard error of the mean (n = 2). Total Minor VFAs, a sum of isobutyric acid, valeric acid; isovaleric acid and hexanoic acid. Species not sharing the same letters in the superscripts within a column are significantly different. Species with the * sign in the superscript within a row are significantly different from the sample of the same species from another harvesting season. Species with the # sign in the superscript are significantly different from maize silage (MS) fermented alone.

ascribed to the higher production of acetic acid by all three species (*A. esculenta:* $P = 0.005$, *F. vesiculosus:* $P = 0.0015$, and *A nodossum:* $P = 0.025$), of butyric acid by *A. esculenta* ($P = 0.0004$) and *F. vesiculosus* ($P = 0.033$), and of minor VFAs (sum of isobutyric acid, valeric acid, isovaleric acid, and hexanoic acid) by *A. esculenta* when harvested in the spring ($P = 0.005$).

When compared with MS fermented alone, only two of the 12 macroalgae: *F. vesiculosus* ($P < 0.033$) and autumn harvested *A. nodosum* ($P = 0.01$), reduced total VFA concentrations in the post-fermentation rumen fluid namely by 37.5 and 21.6%, respectively (Suppl. Figure 3). With *F. vesiculosus*, this was associated with a sharp reduction in concentrations of acetic acid (autumn harvest only, $P < 0.0001$) and, in both species, by decreased concentrations of butyric acid (*F. vesiculosus:* $P < 0.03$; autumn harvested *A. nodosum:* $P = 0.002$). Butyric acid concentrations were, in general, most susceptible to change upon macroalgae addition to MS, and were also significantly decreased by autumn harvested *A. esculenta* ($P = 0.003$) and *F. serratus* ($P = 0.026$). However, none of the macroalgae addition reduced propionate production as compared to pure MS. Instead, the addition of autumn harvested *F. vesiculosus* resulted in a significantly higher propionate:acetate ratio as compared to the pure MS fermented alone ($P = 0.035$) (Suppl. Fig. S4). In addition, seasonal impacts were visible on the propionate: acetate ratio, but only in the autumn harvested species *F. vesiculosus* ($P = 0.038$) and *A. esculenta* ($P = 0.0002$) as compared to those from the spring harvest.

3.4.3. Total gas and methane production

Addition of *A. nodosum* ($P < 0.0001$) and *F. vesiculosus* ($P < 0.0001$), irrespective of the harvesting season, as well as spring harvested *F. serratus* ($P = 0.04$), reduced TGP per gram OM of the feed (macroalgae + MS). The reduction in TGP was most pronounced with autumn harvested samples of *F. vesiculosus* as compared to spring harvested samples ($P = 0.025$). In contrast, for the red macroalga *P. palmata*, there was a lower TGP with the addition of spring harvested material as compared to autumn harvested material ($P = 0.042$) (Table 5).

A significant reduction of CH_4 produced per gram OM of the feed (macroalgae + MS) was achieved with autumn harvested *F. vesiculosus* ($P = 0.0009$) and *A. nodosum* ($P = 0.023$), as well as for spring harvested *C. crispus* ($P = 0.0037$) with ~62.6%, ~48.2%, and ~56.5% reductions, respectively (Table 5, Suppl. Fig. S3). In terms of relative proportions of CH_4 in the produced total gas, the reductions were 50.4%, 47.3%, and 34.8% for *C. crispus* (spring), *F. vesiculosus* (autumn), and *A. nodosum* (autumn) respectively. A >30% numerical reduction in CH_4 production during fermentation was also observed for brown species *F. serratus* and the green species *U. lactuca*, irrespective of the harvesting season, and for spring harvested *P. umbilicalis*, but these reductions did not reach significance.

3.5. Hierarchical clustering of macroalgae based on effects on TGP production

The time course of development in TGP during fermentation suggested that the rate of gas production from MS with or without added macroalgae was minimal at the beginning of the fermentation, but an exponential increase occurred after approximately 15–22 h of fermentation, and a maximal TGP (asymptote) was reached after approximately 36 h (Figs. 2a and 3). When a center scaling across macroalgae species was undertaken followed by hierarchical clustering, macroalgae species clustered into four distinct groups based on their impact on TGP: high gas producer, medium-high gas producer, average gas producer, and low gas producer (Figs. 2b and 3). As observed with other parameters, autumn and spring harvested biomass of macroalgae clustered at the same region in the heatmap, except for *A. esculenta, C. crispus and H. elongata*, indicating a minimal effect of seasonality on TGP. Five

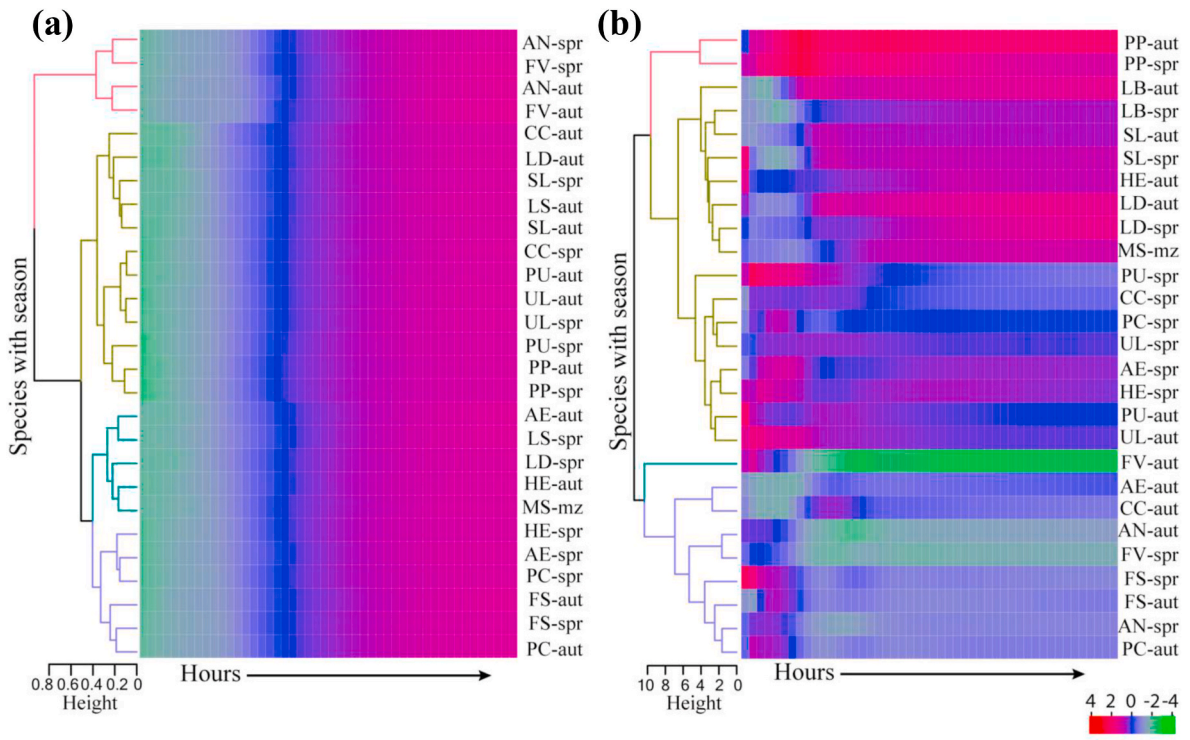

Fig. 2. Clustering of macroalgae based on their effects on total gas production when co-fermented *in vitro* with maize silage in buffered rumen fluid for 48 h. Two batches of *in vitro* fermentations were conducted to simulate rumen fermentation, where macroalgae were added to a standard feed, maize silage, in a 20%-to-80% ratio in dry matter and then incubated for 48-h in a buffered rumen inoculum. Each row represents a species from a specific season, and each column represents the accumulated total gas production after a specific duration (hr) of fermentation. (a) The total gas production values were center-scaled along time (row-wise) followed by a heatmap generation to evaluate gas production status for each hour of fermentation; (b) The total gas values were center-scaled across species (column-wise) followed by heat map generation to cluster macroalgae species. The color scale of the heat map denotes the center-scaled value in the form of color, in which red color indicates the maximum value and green color indicates the minimum value. Also, species from specific seasons were clustered together based on the agglomerative hierarchical clustering algorithm. Here, the values were clustered together row-wise (along time) based on similarity and were represented by a dendrogram. The height of the dendrogram shows the distance or dissimilarity between the two species. The unique colors of the dendrogram indicate the specific clusters. aut, Autumn; spr, Spring; MS-mz, Maize silage incubated alone without addition of macroalgae; AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus serratus;* FV, *Fucus vesiculosus*; HE, *Himanthalia elongata*; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculate*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*.

macroalgae species: *P. palmata* (high gas producer), *S. latissima* (medium-high gas producer), *U. lactuca* (average gas producer), *F. vesiculosus* (low gas producer), and *A. nodosum* (low gas producer) were selected to evaluate their effects on the rumen microbiome.

3.6. Effects of addition of macroalgae to maize silage on the rumen microbiome

The 16S rRNA amplicon sequencing of the rumen microbiome with 44 samples yielded a total of 987665 reads with 22447 ± 4756 clean reads per sample (mean ± standard deviation) with 3026 amplicon sequence variants (ASV). Amongst the 3026 detected ASVs, 3013 ASVs accounted for rumen bacteria, while the other 13 ASVs represented rumen archaea. The addition of macroalgae to MS differentially modulated the rumen microbial composition after 48 h of fermentation. The microbial Shannon α-diversity index was affected differently with the addition of macroalgae ($P = 0.033$, Kruskal Wallis test) (Fig. 4a), and autumn harvested samples tended to reduce this diversity as compared to the spring harvested macroalgae ($P = 0.055$, Kruskal Wallis test). Pairwise comparisons showed that addition to MS of the brown macroalga *F. vesiculosus*, lowered the species richness as compared to the addition of *P. palmata* ($P = 0.021$), *S. latissima* ($P = 0.029$), *U. lactuca* ($P = 0.029$), and also compared to MS fermented in pure form ($P = 0.067$). When macroalgae were clustered based on their effects on bacterial and archaeal β-diversity using PCoA plots, a clear difference among macroalgae was observed, which was in line with their impacts on the other observed characteristics during fermentation of the feeds (Fig. 4b). Relative abundance analyses at the phylum (Fig. 5) and genus (Fig. 6) level revealed that several cellulolytic bacteria belonging to the phylum Firmicutes: *Ruminococcus* 2, Ruminococcaceae UCG-010, Ruminococcaceae NK4A2, Lachnospiraceae XPB1014 groups, as well as Bacteriodetes: Rikenelaceae RC9 gut group (Suppl. Fig. S5a) were inhibited by the two macroalgae, *F. vesiculosus* and *A. nodosum*, which deteriorated fermentability of the basal feed. On the other hand, these two species promoted hemicellulolytic microorganisms, such as *Prevotella* 1, Prevotellaceae NK3B31 group (Bacteriodetes), and *Treponema* 2 (Spirochaetes) in comparison with the post-fermentation rumen fluid derived from the fermentation of MS alone or with the addition of other macroalgae species.

Interestingly, the relative abundance of phylum Euryarchaeota which consists of methanogenic archaea was lowered (Fig. 5), when these four macroalgae species were co-fermented with MS: *F. vesiculosus, A. nodosum, S. latissima,* and *U. lactuca*, and the most dominant methanogenic archaea, *Methanobrevibacter* spp., was significantly reduced by *F. vesiculosus* ($P < 0.025$) (Suppl. Fig. S5b). Macroalgae harvest season did not have any impact in this respect.

3.7. Correlation between chemical composition and rumen fermentation parameters

Overall, a strong inverse correlation was demonstrated between TPC contents of macroalgae and most of the fermentation parameters: OMD

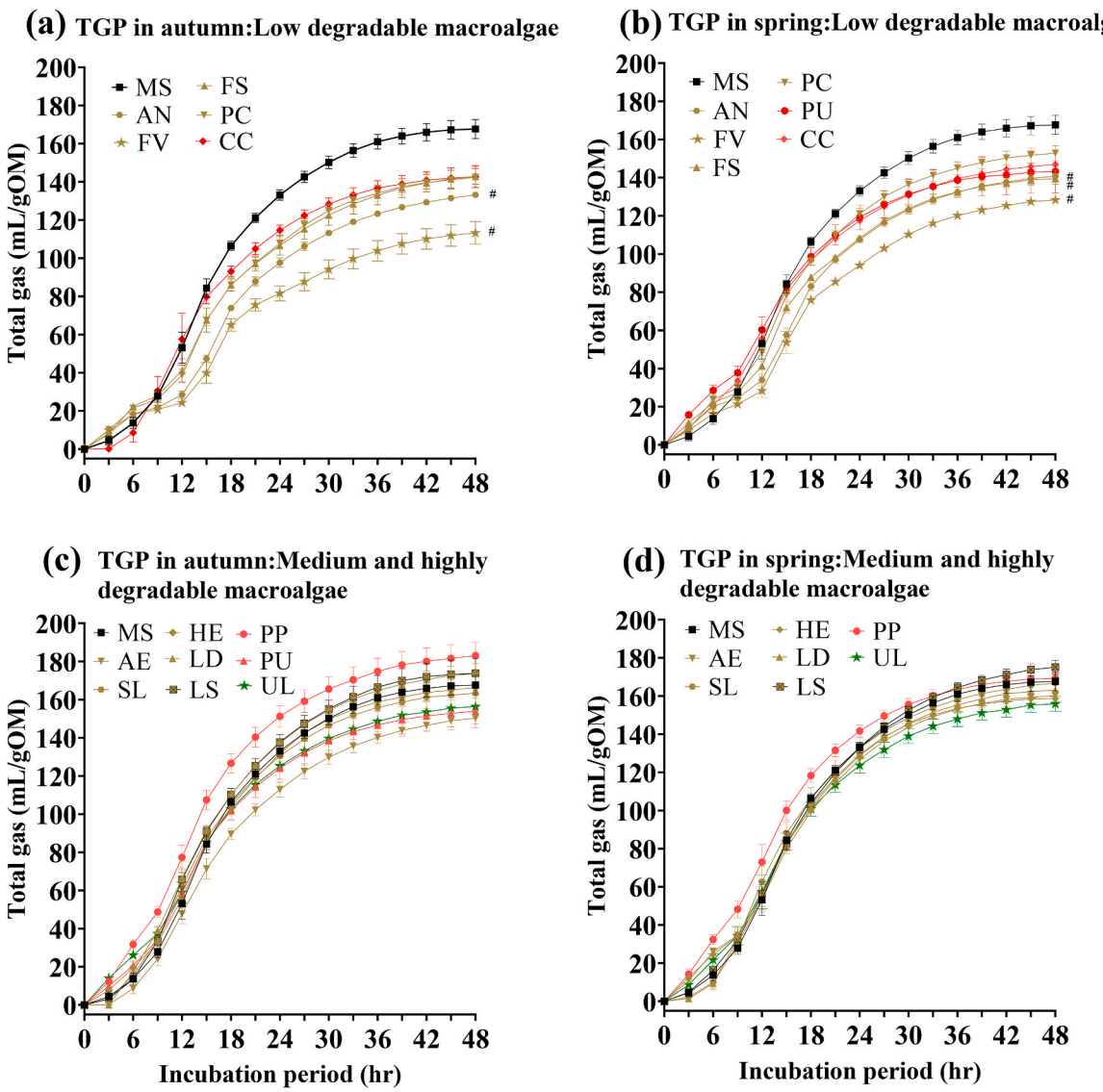

Fig. 3. Effect of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on total gas production. Red, brown, and green colored curves indicate the phylum (type) the macroalgal species belonged to, which was co-fermented with maize silage (MS) in a 20%-to-80% ratio (see legends in Fig. 2). #, Different from MS incubated without macroalgae addition; AE, *Alaria esculenta*; AN, *Ascophyllum nodosum*; FS, *Fucus* serratus; FV, *Fucus vesiculosus*;/gOM, Per gram organic matter; HE, *Himanthalia elongata*; hr, hours; LD, *Laminaria digitata* (blade); LS, *Laminaria digitata* (stipe); PC, *Pelvetia canaliculata*; SL, *Saccharina latissima*; CC, *Chondrus crispus*; PP, *Palmaria palmata*; PU, *Porphyra umbilicalis*; UL, *Ulva lactuca*.

(r = −0.85; $P < 0.001$), TGP (r = −0.79; $P < 0.001$), total VFA concentration (r = −0.78; $P < 0.001$) and CH_4 production (r = −0.53; $P < 0.03$). Crude protein content of macroalgae was moderately and positively correlated to concentrations of the minor VFAs, such as isobutyric acid, valeric, and isovaleric acid (r = 0.53 to 0.60; $P < 0.05$) in the fermented liquid and to OMD (r = 0.39; $P = 0.002$) (Fig. 7).

The Spearman's correlation analyses confirmed an expected negative correlation between TPC in macroalgae to the microbial Shannon Index (r = −0.46, $P = 0.029$) (Suppl. Fig. S5c) and abundance of fiber degrading microorganisms, such as Ruminococcaceae UCG-010 (r = −0.79, $P < 0.0001$), Rikenellaceae RC9 gut group (r = −0.71, $P < 0.0001$), and *Saccharofermentans* (r = −0.75, $P < 0.0001$), and a positive correlation to abundance of *Prevotella* spp. (r = 0.89, $P < 0.0001$), *Ruminobacter* (r = 0.76, $P < 0.0001$) and *Treponema* 2 (r = 0.70, $P = 0.0001$) (For details: Appendix A, Suppl. data SD1). However, no significant correlation of TPC against *Methanobrevibacter* spp. could be detected.

4. Discussion

The major hypotheses of this study were that a) Nordic macroalgae harvested in the spring are more suitable to be utilized as ruminant feed resources than those harvested in the autumn due to their more favorable nutritional values and influences on rumen degradability, and b) polyphenol-rich brown macroalgae are more capable of reducing enteric CH_4 production, irrespective of the harvesting season, due to the polyphenol-associated changes in the rumen microbiome and subsequent rumen fermentation characteristics.

The major findings of this study were that: a) macroalgae chemical compositions differed in a phylum-specific manner, but brown macroalgae possessed greater seasonal variability in ash, protein, and total polyphenol content than red and green species, b) impacts of addition of macroalgae to the standard feed MS on *in vitro* ruminal feed degradation and CH_4 production were species-specific, and only two brown species, *F. vesiculosus* and *A. nodosum*, harvested in autumn and a red species, *C. crispus*, harvested in spring appeared to have significant anti-methanogenic properties (~48–62.6% CH_4 reduction), and c) TPC

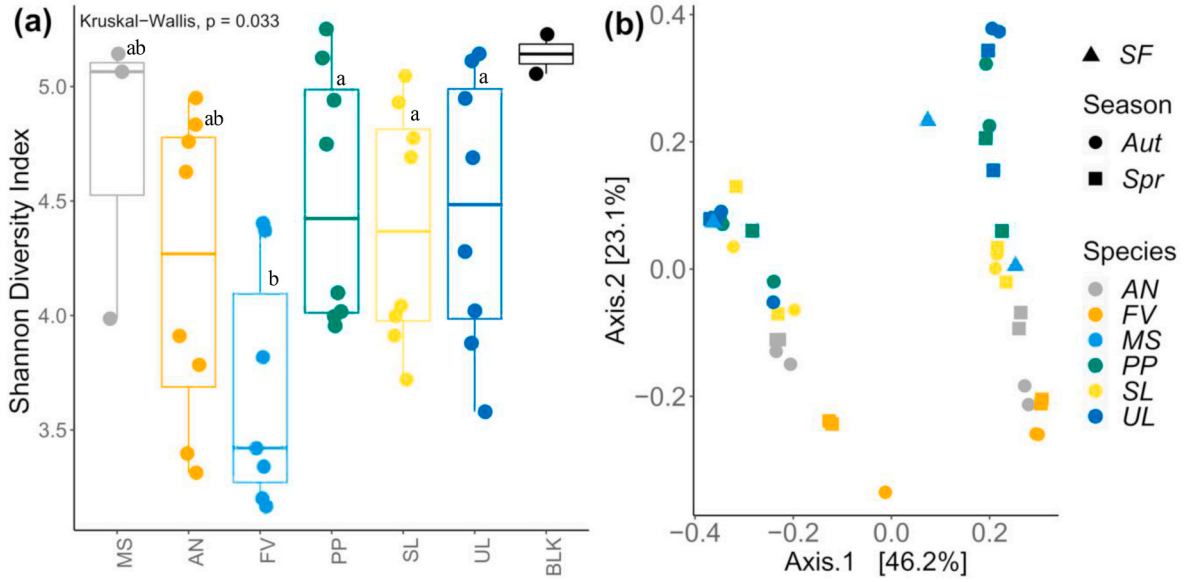

Fig. 4. Effects of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on the rumen microbiome. (a) Alpha diversity estimated by Shannon diversity matrices; (b) Beta diversity based on Bray-Curtis dissimilarity matrix visualized using Principal Coordinate Analysis. Macroalgae were co-fermented in buffered rumen fluid with the standard feed (SF) maize silage in a 20%-to-80% ratio on a dry matter basis (see legends to Fig. 2). AN, *Ascophyllum nodosum*; Aut, Autumn; BLK, Blank samples incubated with buffered rumen fluid alone without maize silage or macroalgae; FV, *Fucus vesiculosus*; MS, Maize silage incubated without macroalgae; PP, *Palmaria palmata*; SL, *Saccharina latissima*; Spr, spring; UL, *Ulva lactuca*. In Fig. 4 (a), blank samples are presented as a reference but were not included in the statistical analysis. Species not sharing the letters over the box plots are significantly different.

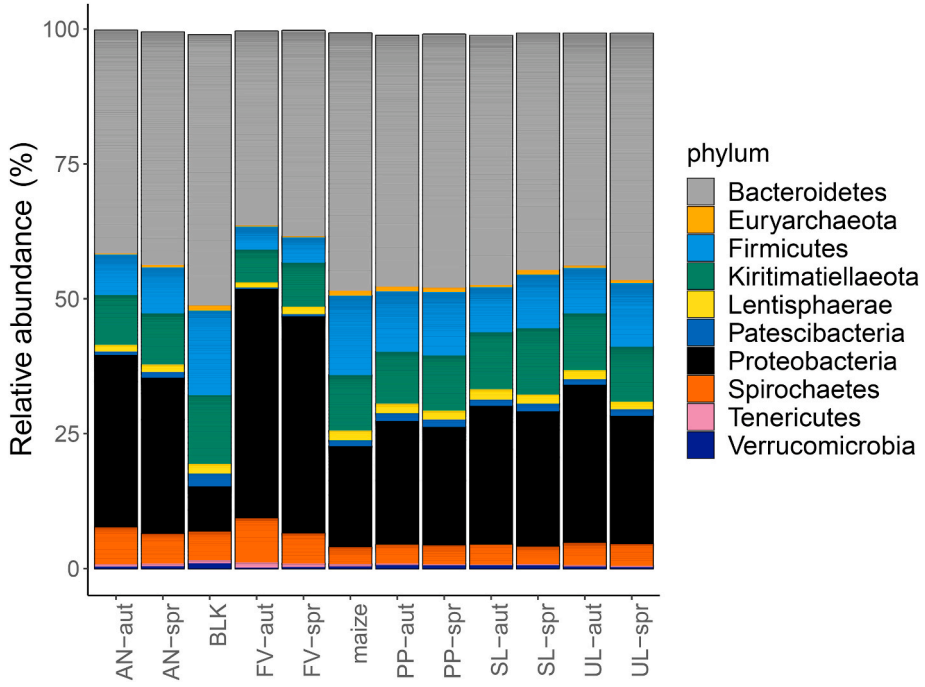

Fig. 5. Effects of co-fermenting macroalgae *in vitro* with maize silage in buffered rumen fluid for 48 h on the relative abundance of the rumen microbiome at the phylum level. AN, *Ascophyllum nodosum*; aut, Autumn; BLK, Blank samples incubated with buffered rumen fluid alone without maize silage or macroalgae; FV, *Fucus vesiculosus*; MS, Maize silage incubated without macroalgae; PP, *Palmaria palmata*; SL, *Saccharina latissima*; spr, spring; UL, *Ulva lactuca*.

content of macroalgae seemed to be a major determining factor behind the suppression of feed degradability and CH_4 production, directly affecting the diversity and abundance of rumen fibrolytic bacteria and indirectly the methanogens.

4.1. The potential of macroalgae as ruminant feed additives and their interspecies and seasonal variations

Our findings suggested that the chemical composition (CP, NDFom, mineral elements, and TPC) of macroalgae are predominantly determined by their phyla. Overall, red (*C. crispus, P. palmata,* and *P. umbilicalis*) and green (*U. lactuca*) species had the highest protein

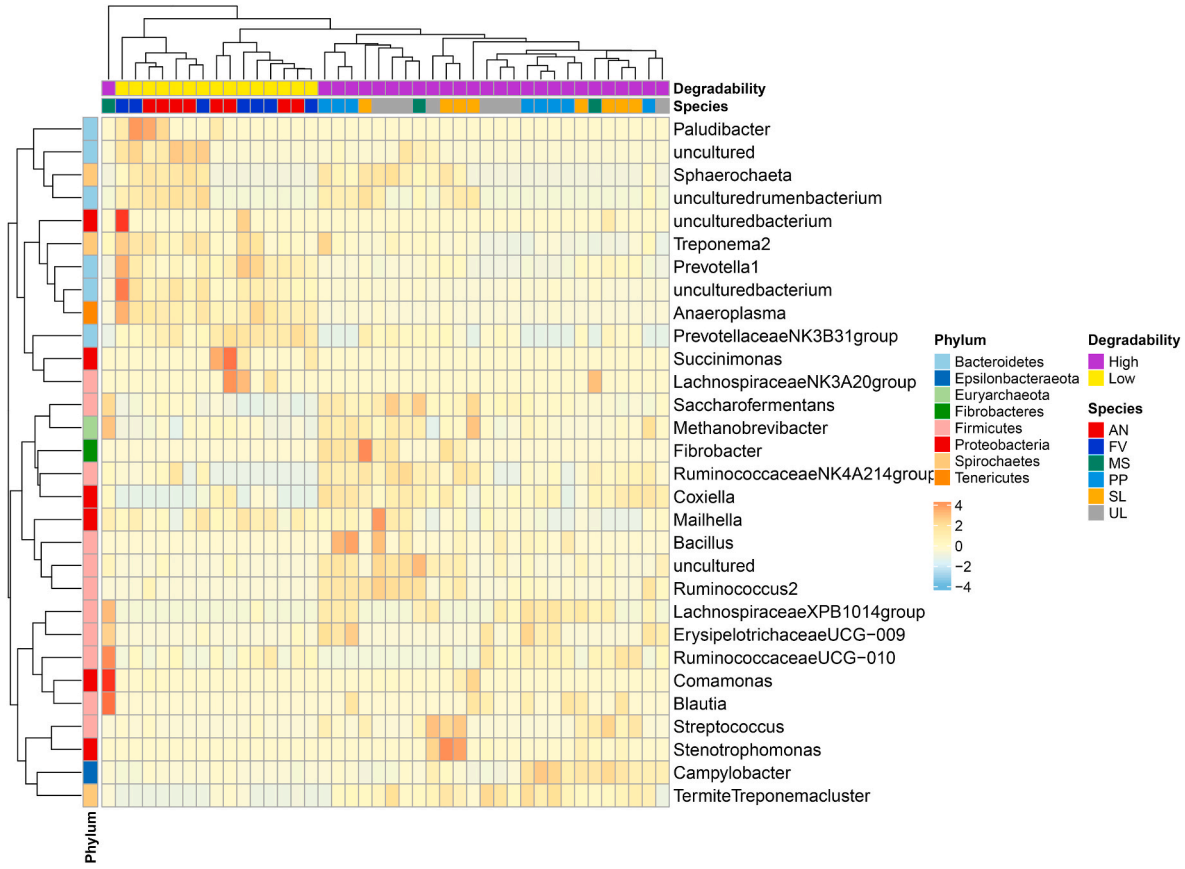

Fig. 6. Heat map showing abundance of significantly affected microbial taxa and genera of rumen microbiome after co-fermenting macroalgae with maize silage in buffered rumen fluid for 48 h *in vitro*. MS, Maize silage; AN, *Ascophyllum nodosum*; FV, *Fucus vesiculosus*; SL, *Saccharina latissima*; PP, *Palmaria palmata;* UL, *Ulva lactuca*; BLK, Blank; aut, Autumn; spr, Spring. See legends in Fig. 2 for further details.

contents (up to 20% CP of DM) in agreement with previous findings (Gaillard et al., 2018; Mæhre et al., 2014). Among brown macroalgae, a few species (e.g., *A. esculenta* and *S. latisima)* with a medium CP level (8–13% of DM) could have better value as protein feed resources than other brown species, such as fucoids. Macroalgal proteins have been characterized as quality proteins comprised of a high proportion of essential amino acids (~46% of total amino acids) (Angell et al., 2016a). The digestibility of macroalgal CP is generally found to be higher for red and green species (64–87%) than for brown species (55–82%), but there are large interspecies variations within phyla (Gülzari et al., 2019; Tibbetts et al., 2016). Certain red (*Porphyra* sp., *P. palmata*) and green (*Cladophora* sp., *Ulva* sp.) species have shown higher ruminal as well as total tract degradability of amino acids compared to brown species such as *L. digitata* in an *in sacco* study (Gaillard et al., 2018). Hence, large interspecies variations among macroalgae have been found *in situ* in dairy cattle with regards to total tract digestibility of CP, due to differences in both rumen degradability and intestinal digestibility of proteins escaping rumen fermentation (Tayyab et al., 2016). These findings suggest that in the search for novel protein feed resources, red and green macroalgae would be the most promising candidates due to favorable CP contents and amino acid compositions and highest digestibility.

In this research, a variable part of DM of macroalgae was identified as NDFom (16–62%), which generally represents material that cannot be degraded by enzymes produced by the animal itself. Hence, rumen degradability of NDF is a major determining factor for the overall digestibility and value of macroalgae as feeds for ruminants. There is limited information available from *in sacco* (goat) and *in vivo* (sheep) studies on this matter. These studies have suggested that only 16–29% of NDF from species such as *U. lactuca*, *Ulva rigida,* and *Gracilaria vermicophylla* is digestible to ruminants (Ana et al., 2017; Ventura and Castañón, 1998). Thus, the low digestibility of macroalgae fiber that results in overall low DM or OM digestibility could be one of the major bottlenecks of using larger proportions of the macroalgae in the feeds for high producing ruminants (Ana et al., 2017). However, in the present study, when certain red (*P. palmata* and *P. umbilicalis* from spring), brown (*S. latisima, L. digitata, A. esculenta* from spring, *H. elongata*) and green (*U. lactuca*) macroalgae were added to MS, no significant changes of the overall OMD *in vitro* occurred. This agrees with a previous *in vitro* 8-day rusitec fermentation study with 25% DM inclusion (as compared to 20% in the present study) of *G. vermiculophylla, S. latissima,* and *Ulva rigida* to a total mixed ration comprised of haylage, corn silage, wheat straw, and a commercial concentrate (Maia et al., 2019). The rest of the brown and red (*C. crispus*) species of macroalgae in the current study lowered the overall OMD by 12–25%, and these negative effects on OMD may be ascribed to their high contents of poorly degradable complex polysaccharides (alginates, fucoidan, carrageenan) (Williams et al., 2013) as well as the contents of other macroalgal compounds that may interfere with the bacterial fermentation.

Macroalgae are known to contain high levels of a range of minerals, and contents are generally higher than in terrestrial plants (Rupérez, 2002). In this study, the brown species *H. elongata,* and *L. digitata* were mainly enriched with the essential macrominerals Ca, Mg, Na, and K (also for *S. latissima)* compared to other species. Similarly, green species, *U. lactuca* was enriched with Na and Mg. Thus, macroalgae included in this study could be considered as potential sources of macrominerals for ruminants. The inclusion of intact macroalgae as a significant proportion of diets can, however, be of concern, as the dietary maximum tolerable levels of certain minerals, such as sodium chloride (1 g salt/kg body weight), iodine (50 mg/kg diet), arsenic, fluorine, cadmium, etc. may be exceeded (Bikker et al., 2020; NRC, 2005). Thus, specific post-harvest

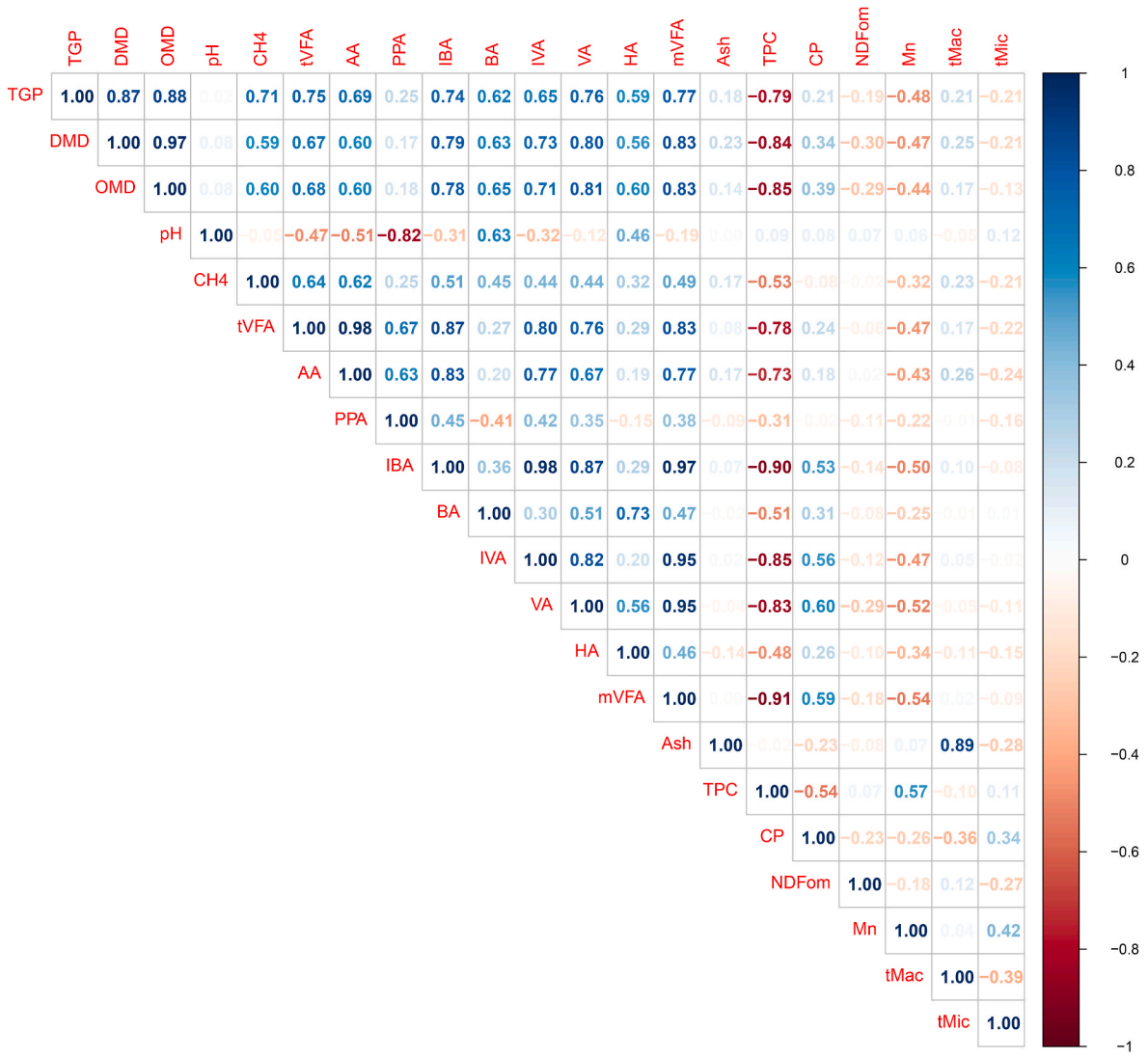

Fig. 7. Correlation matrix between the chemical composition of macroalgae and the *in vitro* fermentation characteristics after their co-fermentation with maize silage in buffered rumen fluid for 48 h. TGP, Total gas produced per gram of organic matter; DMD, dry matter degradability; OMD, Organic matter degradability; pH, Fermentation pH; CH_4, Methane; tVFA, Total volatile fatty acids; AA, Acetic acid; PPA, Propionic acid; BA, Butyric acid; IBA, iso-butyric acid; VA, valeric acid; IVA, isovaleric acid; HA, hexanoic acid; mVFA (sum of IBA,VA, IVA, and HA); TPC, Total polyphenol content; CP, Crude protein; NDFom, Ash corrected neutral detergent fiber; Mn, Manganese; tMac, Total macrominerals; tMic, Total microminerals. See legends in Fig. 2 for further details.

processing techniques, such as blanching, of macroalgae may be necessary to reduce the contents of critical minerals before their inclusions in the diets (Nielsen et al., 2020).

In addition to protein, fiber, and minerals, macroalgae are known to contain bioactive compounds, of which e.g. polyphenols have been associated with health-promoting effects (Ford et al., 2020; Gupta and Abu-Ghannam, 2011). Brown macroalgae, particularly species belonging to the Fucaceae family (*F. vesiculosus*, *A. nodosum*, *F. serratus,* and *P. canaliculata*), were found to be rich in such polyphenolic compounds, and they play an important role in the macroalgal defense against external stressors (Steevensz et al., 2012). Species-specific variations in macroalgal polyphenol contents can be linked to their growth stage, habitats, and exposures to certain biotic (epiphytes, herbivores, microbes, etc.) and environmental stressors (UV-light, salinity, tides) (Connan et al., 2004; Parys et al., 2009). Within brown macroalgae, the species that were collected from the upper (*P. canaliculata*) or middle (*A. nodosum, F. vesiculosus*, *F. serratus)* intertidal zones in Norway had higher TPC levels than species collected from the lower intertidal (*A. esculenta, H. elongata*) and subtidal (*L. digitata, S. latissima*) zones. On the other hand, intertidal zones and macroalgal polyphenol levels were not related in the red or green species, suggesting that they may rely on different biological mechanisms or chemical compounds to deal with external stressors (Roleda et al., 2019; Wada et al., 2015). Therefore, brown species, particularly the fucoids would be the species of interest as a source of natural bioactives for the health benefits of ruminants.

In agreement with previous studies including from the Nordic region (de la Moneda et al., 2019; Tayyab et al., 2016), seasonal changes in macroalgae compositions were observed also in this study. Specifically, spring harvested macroalgal biomass had the highest CP and mineral contents, whereas the highest TPC levels were found in autumn harvested biomass in some brown species. The seasonal variations were substantial for some species (54–96% higher contents of minerals in spring harvested: *S. latissima, L. digitata* (blade), and *P. palmata;* 66–86% higher CP contents in spring harvested: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata*; 23–49% higher TPC contents in autumn harvested: *A. esculenta, P. cannliculata,* and *F. vesiculosus*). The higher CP contents in spring harvested macroalgae can be attributed to more favorable growing conditions in the spring, as evidenced by higher nitrogen and dissolved oxygen in seawater as well as increased light intensity, all of which can favor nutrient uptake and assimilation (Gaillard

et al., 2018; Rødde et al., 2004). In the autumn, more pronounced signs of biofouling and biomass deterioration were observed in the macroalgae, as also reported by others (Lüning, 1993; Stévant et al., 2017). This can probably explain, why some brown species had the highest level of TPC in the autumn, since polyphenols as mentioned earlier play an important part in the macroalgae defense against external stressors.

4.2. Impacts of inclusion of macroalgae on in vitro fermentation characteristics of feed and the role of total polyphenol contents

The present study implies that species-specific modulations of rumen fermentation characteristics (OMD, TGP, VFA, and CH_4) occur, when macroalgae are added to a standard feed (MS), and the level of TPC in macroalgae appears to be the principal explanatory factor behind such modulations. Hence, with the addition of species containing low TPC and generally high levels of CP (*P. palmata, L. digitata, S. latissima*, *H. elongata*, *A. esculenta,* and *U. lactuca*) to MS, the *in vitro* fermentation characteristics remained similar to those of MS fermented alone. This was irrespective of the harvesting season, except for *A. esculenta*, where VFA production and OMD were reduced upon the addition of autumn harvested material. This suggests that species showing no obvious negative impacts on rumen fermentation patterns could constitute up to 20% of a ruminant diet without undesirable effects on rumen fermentation. On the other hand, when brown macroalgae, especially from the Fucaceae family: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata* were added to MS, a reduction of the overall OMD resulted. Additionally, *A. nodosum* and *F. vesiculosus* diminished TGP and VFA production (24–37.5%). These fucoid species are characterized by high contents of TPC and relatively low CP levels. The negative impacts of *F. vesiculosus and A. nodosum* on feed degradation parameters were more pronounced, when they had been harvested in the autumn, and this was associated with lowered production during fermentation of acetic acid and/or butyric acid, which are important parts of the energy supply to ruminant animals. Phlorotannins, the major constituents of polyphenols in brown macroalgae, form complexes with protein molecules by non-covalent bondings, thereby impairing the degradation of dietary CP and presumably also the activity of extracellular bacterial enzymes (Vissers et al., 2018; Wang et al., 2008). They may additionally inhibit microbial attachment to fiber materials, thus reducing the efficiency of NDF degradation (Makkar, 2003; Wang et al., 2008). Thus, brown species from the Fucaceae family are not suitable for inclusion in ruminant diets in as high proportions as used in this study due to the potential negative impacts of high polyphenol contents on feed utilization and associated animal productivity.

Our preliminary data (not provided) indicated that simple pretreatments of fresh macroalgal biomass can reduce the TPC content by up to 25% in species from the Fucaceae family. Such TPC-reduced material, when added (20%) to MS gave rise to ~25% higher TGP than the native material (Deepak et al., unpublished data). Therefore, extraction of polyphenols from TPC rich macroalgae via novel biorefinery approaches, such as microwave, ultrasound, or enzyme-assisted extraction, may be a feasible way to overcome the negative impacts of high TPC on feed degradability, while recovering the highest amount of bioactive polyphenols for other purposes (Filote et al., 2021; Marinho et al., 2016).

4.3. Impacts of Nordic macroalgae on the rumen microbiome and potential anti-methanogenic properties

The composition of the rumen microbial population plays an important role in the degradation of dietary plant components (Mizrahi et al., 2021). In this study, the rumen microbial compositions were differentially modulated by the addition of macroalgae species to MS. The effect of the macroalgae harvesting season in this respect was marginal. Some macroalgae species (*P. palmata, S.latissima,* and *U. lactuca*) that did not have apparent effects on rumen fermentation parameters or *in vitro* feed degradation, induced only minimal changes in microbial compositions in the post-fermentation rumen fluid as compared to MS fermented alone. In contrast, supplementation of *A. nodosum* and *F. vesiculosus* to MS was associated with an overall reduction in rumen microbial species richness (18–21%) and diversity (Shannon diversity index, 4.72 vs 3.67: MS vs *F. vesiculosus*). These two macroalgae species substantially inhibited the abundance of fiber degrading cellulolytic bacteria belonging to the taxa Firmicutes: *Ruminococcus* spp., Ruminococcaceae UCG-010, species in the Lachnospiraceae family, and Bacteriodetes: Rikenelaceae RC9 gut group. This had implications in terms of a reduced OMD, presumably in part due to reduced cellulose degradability, since the abundance of cellulolytic bacteria in this study was directly correlated with rumen fermentation parameters. In contrast to our results, a few *in vitro* (Belanche et al., 2016a) and *in vivo* (Zhou et al., 2018) studies have reported only minor changes in rumen microbial populations, when *A. nodosum* was added to a basal feed. This could probably be due to a lower level of macroalgal inclusion (≤5% DM) in the diet than in the present study. In this study, although cellulolytic bacteria were suppressed, the addition of *A. nodosum* and *F. vesiculosus* to MS, promoted hemicellulolytic bacteria, such as *Prevotella* spp. (Bacteriodetes), and *Treponema* 2 (Spirochaetes) indicating the microorganism-specific effects of macroalgae. These effects are analogous to the impacts of *A. nodosum*-derived phlorotannin on cellulolytic and non-cellulolytic rumen microorganisms as reported in a previous *in vitro* study (Wang et al., 2009). The promotion of rumen hemicellulolytic microorganisms by the polyphenol-rich macroalgal feed may be an indication of a compensatory enhancement of hemicellulolytic activity in response to poor cellulose degradation. However, further studies regarding the impacts of polyphenol-rich macroalgae on different fiber degradation rates are required to confirm this hypothesis.

The macroalgae species included in this study represented a range of CH_4 mitigation potential when they were added to the MS. Such mitigation was >30% per gram of fermented OM with brown species: *F. vesiculosus*, *A. nodosum,* and *F. serratus*; green species: *U. lactuca* regardless of harvesting seasons, and the red species: *C. crispus* and *P. umbilicalis* from the spring harvest. However, the most promising CH_4 mitigation was observed for poorly degradable fucoid species, namely 48.2% and 62.6% reduction, respectively, for *A. nodosum,* and *F. vesiculosus* from the autumn harvest, and ~56.5% for the red species, *C. crispus* from the spring harvest. To the best of our knowledge, such anti-methanogenic action of *F. vesiculosus* and *C. crispus* has never been reported. The potency of these Nordic macroalgae species encourages to further *in vivo* studies to explore their potential application as anti-methanogenic feed additives for ruminants.

The anti-methanogenic property of *A. nodosum* has previously been reported from an *in vitro* study that used the rusitec system. In that study, only a 15% reduction in CH_4 production per gram of fermentable OM was observed when 2 g L^{-1} *A. nodosum* was added to the fermentation medium (Belanche et al., 2016b). Two tropical red species, *Asparagopsis taxiformis* and *A. armata* are well documented to possess very potent anti-methanogenic actions, and they can almost entirely reduce CH_4 production from ruminant livestock both *in vitro* (Machado et al., 2014) and *in vivo* (Kinley et al., 2020; Roque et al., 2019b) at low levels of dietary inclusion (<5% of OM). In addition, variable anti-methanogenic properties *in vitro* have been ascribed also to the tropical brown macroalgae *Dictyota bartayresii* (Machado et al., 2014) and the red *Gracilaria vermiculophylla* and *Gigartina* sp., as well as the widely distributed green *Ulva* sp. (Maia et al., 2016). The CH_4 mitigating properties of selected brown macroalgae in our research were associated with a marked reduction in the abundance of the dominant CH_4 producing archaea, *Methanobrevibacter* (phylum Euryarchaeota) in the post-fermentation rumen fluid. These results are in agreement with previous studies that evaluated the impacts of *A. nodosum* (Zhou et al., 2018) and *A. taxiformis* inclusion on rumen methanogenic archaea (Machado et al., 2018; Roque et al., 2019a). Therefore, certain Nordic brown and red macroalgae species can mitigate enteric CH_4 formation from ruminants by inhibiting

the methane-producing archaea in the rumen.

The reduced abundance of rumen methanogens by the red macroalgae *Asparagopsis* spp. has been ascribed to direct inhibitory properties of halomethanes, such as bromoform and dibromochloromethane on the rate-limiting enzymatic process, where CH_4 is formed (Machado et al., 2018; Roque et al., 2019a). However, a recent study with 17 different red, brown and green species (including *A. nodossum*, *F. vesiculosus* and *C. crispus*) indicated that such halomethanes are not present in Nordic macroalgae (Nørskov et al., 2021). Thus, other secondary polyphenolic metabolites, such as phlorotannin and flavonoids, could be responsible for the CH_4 mitigating properties of brown (Vissers et al., 2018; Wang et al., 2008) and red species (*C. crispus*), respectively (Bodas et al., 2008). Surprisingly, no negative correlation between macroalgae TPC and rumen methanogens (*Methanobrevibacter* spp.) was evident in this study, suggesting that alternative bioactive compounds or mechanisms may be involved with a direct or indirect suppression of methanogenesis as well as the population of methanogens.

Rumen microorganisms exist in a complex system where the activities of different microbial species can be interconnected. Certain rumen methanogenic archaea live in a symbiotic relationship with ciliated protozoa where they utilize H_2 produced by the protozoa for methanogenesis and energy metabolism (Patra et al., 2017). The addition of 2 g L^{-1} A. *nodosum*, a phlorotannin-rich macroalga, in the fermentation medium suppressed the CH_4 production and rumen protozoa (by 23%) with a lowered acetate and butyrate production, presumably due to an anti-protozoal effect of phlorotannins (Belanche et al., 2016b). A reduction in the ciliated protozoa and CH_4 production without affecting rumen methanogens was observed in another *in vitro* batch fermentation study when ethanolic extracts of two brown macroalgae, *Undaria pinnatifida*, and *Sargassum fulvellum*, containing diverse phenolic compounds (flavonoids and polyphenols) were added to a control diet (Choi et al., 2021). Thus, the inhibition of rumen CH_4 production by polyphenol-rich macroalgae in this study may be associated with indirect modulation of the rumen environment potentially due to reduced rumen protozoal activity as supported by the observed reduction in acetate and butyrate production. The rumen protozoal population has also been found to be correlated positively with the concentrations of acetic acid and negatively with that of propionic acid (Zhou et al., 2018). We observed an increased propionate:acetate ratio with the addition of *F. vesiculosus* (autumn harvested) to MS, suggesting a potential reduction in protozoal activities and subsequently, contributing to CH_4 mitigation. However, studies directly evaluating the impacts of phlorotannins on the rumen protozoa and methanogens are lacking. Therefore, future studies should evaluate not only the specific effect of phlorotannins on methanogenesis and the populations of rumen methanogens and protozoa, but also investigate whether there are other active anti-methanogenic components (e.g. flavonoids, complex carbohydrates) that could be involved in the suppression of CH_4 production by these brown macroalgae.

To date, *Asparagopsis* spp. are the most potent macroalgae in terms of mitigating CH_4 emissions from ruminant livestock. However, scalable commercial cultivation of *Asparagopsis* spp. to produce sufficient biomass to be used in livestock farms is yet to be achieved due to its complex life cycle and requirements for optimal growth in an artificial environment (Zhu et al., 2021). Moreover, *Asparagopsis* spp. are not the native species in Nordic waters (Andreakis et al., 2004) and there are safety concerns due to the potential toxic properties of the major bioactive compound, bromoform, for animals and the environment (Muizelaar et al., 2021). In contrast, a large volume of biomass of the brown macroalgae: *A. nodosum* and *F. vesiculosus* are naturally available across the North Atlantic seacoasts all year around (Pereira et al., 2020; Stévant et al., 2017), although commercial cultivation is yet to be established for these species. Nonetheless, our findings suggest that certain Nordic macroalgae could serve as ingredients for the production of anti-methanogenic feed additives, with potentially safer bioactive compounds for CH_4 mitigation from ruminants.

Despite the promising CH_4 mitigation, *A. nodosum* and *F. vesiculosus* significantly impaired the OMD, TGP, and VFA production and that could lead to reduced animal productivity if the dietary inclusion rates are too high. Though the rate of CH_4 inhibition was greater than the inhibition of feed degradation, the positive correlation of CH_4 inhibition with the feed degradability inhibition indicates that a part of CH_4 mitigation was due to the reduced feed degradation. Thus, the anti-methanogenic potential of these macroalgae should be validated *in vivo* to design optimal strategies for implementation. In the future, it would be interesting to evaluate whether the anti-methanogenic properties of these macroalgae also persist at lower inclusion levels so that feed degradability is not or minimally compromised. Moreover, using a mixture of highly anti-methanogenic macroalgae species together with less potent species that do not depress rumen feed degradability could be another viable option. Future studies should also focus on the use of polyphenolic or other types of bioactivity enriched fractions, rather than whole biomass, to identify specific compounds directly responsible for anti-methanogenic properties and their mechanisms of action. Certain Nordic species, such as the green macroalga, *U. lactuca,* seem attractive candidates to achieve ~30% of CH_4 mitigation *in vitro* without affecting the feed digestion and animal productivity at 20% inclusion in the feed. Hence, this study points to the importance of certain Nordic macroalgae as potential anti-methanogenic feed additives and/or nutritional feedstuffs for ruminants.

5. Conclusions

Brown (*L. digitata, S. latissima, H. elongata*), red (*P. palmata*) and green (*U. lactuca*) species with low TPC contents could become valuable future feed sources for ruminants, particularly when harvested in the spring, where protein contents are highest. *In vitro,* they could constitute a significant part of the diet (20% of DM) without negative implications for rumen fermentability of the feed or the rumen microbiome. Macroalgae among the brown species with high levels of TPC: *A. nodosum, F. vesiculosus, F. serratus,* and *P. canaliculata,* seem unsuitable for ruminant feed applications at large inclusions due to negative impacts on OMD, fermentation patterns, and the rumen microflora, particularly when harvested in the autumn, where TPC levels are highest. The substantial depression of feed degradability by polyphenol-rich macroalgae: *A. nodosum* and *F. vesiculosus* was associated with depression of cellulose-degrading microorganisms, including *Ruminococcus* spp., Ruminococcaceae UCG-010, species in the Lachnospiraceae family, and Rikenelaceae RC9 gut group. Nevertheless, *A. nodosum* and *F. vesiculosus* could be promising as ingredients in feed additives to mitigate the enteric CH_4 emissions from ruminants through inhibition of methanogens (*Methanobrevibacter* spp.), an effect which could not solely be described by TPC. Future research should validate these findings via *in vivo* feeding trials with ruminants to discover a minimum effective dose of inclusion into the feed from both anti-methanogenic and digestibility perspectives. To optimize a strategy for implementation of macroalgae as feed additives to reduce enteric CH_4 emission, future studies should focus on enrichment of polyphenolic and/or other bioactivity enriched extractions. Therefore, macroalgal biorefinery platforms should identify efficient measures to cost-efficiently extract valuable and safe bioactive compounds that can be utilized for CH_4-mitigating purposes without impairing the digestibility of the diet and hence animal performance.

CRediT authorship contribution statement

Deepak Pandey: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Hanne Helene Hansen:** Resources, Methodology, Data evaluation, Writing – review & editing. **Rajan Dhakal:** Methodology, Data curation, Writing – review & editing, evaluation. **Nabin Aryal:** Methodology, Writing – review & editing. **Surya Prakash Rai:** Visualization, Writing – review & editing. **Rumakanta Sapkota:** Formal analysis, Visualization,

Writing – review & editing. **Mette Olaf Nielsen:** evaluation, Methodology, Writing – review & editing. **Margarita Novoa-Garrido:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Prabhat Khanal:** Conceptualization, Resources, Methodology, Data evaluation, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Abbreviations/nomenclature

AE: Alaria esculenta
AN: Ascophyllum nodosum
AOAC: Association of Official Analytical Chemists
ASVs: Amplicon sequence variants
Ca: Calcium
CC: Chondrus crispus
CH_4: Methane
CP: Crude protein
Cu: Copper
DF: Dilution factor
DM: Dry matter
Fe: Iron
FS: Fucus serratus
FV: Fucus vesiculosus
GC: Gas chromatography
HE: Himanthalia elongata
K: Potassium
LD: Laminaria digitata (blade)
LS: Laminaria digitata (stipe)
Mg: Magnesium
Mn: Manganese
MS: Maize silage
Na: Sodium
NDF: Neutral detergent fiber
NDFom: Ash corrected neutral detergent fiber
OM: Organic matter
OMD: Organic matter degradability
PC: Pelvetia canaliculata
PCR: Polymerase chain reaction
PGE: Phloroglucinol equivalents
PP: Palmaria palmata
PU: Porphyra umbilicalis
rRNA: Ribosomal RNA
SL: Saccharina latissima
SV: Solvent volume
TGP: Total gas production
UL: Ulva lactuca
mVFA: Minor volatile fatty acids
VFA: Volatile fatty acids
Zn: Zinc