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ORIGINAL ARTICLE

Influence of seaweed species and harvest season on in vitro rumen fermentation and methane production with a pasture-based substrate

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ABSTRACT. This study aimed to evaluate the effects of Chilean brown and red seaweeds on *in vitro* rumen fermentation, methane (CH_4) production, and bacterial community structure using a pasture-based substrate. Seven treatments were tested in an *in vitro* fermentation system using rumen fluid collected from Holstein-Friesian cows under a randomised block design, including three seaweed species (brown: *Lessonia spicata* [Ls] and *Macrocystis pyrifera* [Mp]; red: *Gracilaria chilensis* [GCh]), two harvest seasons (winter [WIN] and summer [SUM]), and a control treatment consisting of freeze-dried permanent pasture and concentrate mixed at an 80:20 ratio. Seaweeds were included at 10% of dietary dry matter, replacing part of the concentrate fraction of the substrate. Gas pressure was measured at 2, 4, 6, 8, 10, 12, 24, 36, and 48 h; CH_4 was measured at 4, 24, and 48 h; and ammonia (NH_3) and short-chain fatty acids (SCFA) were analysed at 4 and 48 h. Contrasts were used to evaluate the control treatment vs. seaweed treatments (CTR-SW), comparisons between brown seaweed species (*L. spicata* vs. *M. pyrifera*; LS-MP), comparison between the red seaweed GCh and brown seaweed Mp (*G. chilensis* vs. *M. pyrifera*; GCH-MP), and winter vs. summer harvest seasons (WIN-SUM), where sentence-case abbreviations (e.g., Mp, Ls, and GCh) refer to seaweed species and uppercase abbreviations denote the planned contrasts. Compared with the control treatment (CTR-SW), seaweed treatments reduced NH_3 concentration at 48 h ($P = 0.01$) and CH_4 production at 24 and 48 h ($P < 0.01$). Among the brown seaweeds (LS-MP), Mp resulted in 19% and 13% lower NH_3 than Ls and GCh, respectively, and the lowest total gas and CH_4 production ($P < 0.01$). Relative to GCh (GCH-MP), Mp increased propionate concentration and reduced the acetate:propionate ratio by 12.9% ($P < 0.01$). Although the control treatment (CTR-SW) showed higher total SCFA and nutrient digestibility ($P < 0.01$), harvest season (WIN-SUM) did not affect fermentation or CH_4 production (expressed as a percentage of total gas and mL g OM^{-1}). Denaturing Gradient Gel Electrophoresis Analysis revealed distinct bacterial clustering for Mp (winter) and Ls (both seasons). Overall, Mp reduced *in vitro* ruminal CH_4 and NH_3 concentrations; however, these effects were accompanied by a decrease in digestibility, indicating a trade-off between mitigation and fermentation efficiency. Under the evaluated conditions, none of the seaweeds reduced CH_4 or NH_3 without negatively affecting fermentation, thereby limiting their applicability as mitigation strategies.

Keywords: ammonia, enteric methane, greenhouse gas, macroalgae, phlorotannins, rumen microbiota.

INTRODUCTION

Livestock production systems are a significant source of greenhouse gas (GHG) emissions, contributing approximately 14% of the total anthropogenic emissions (Grossi *et al*., 2018). Methane (CH_4) is the main GHG emitted by ruminants (44% of total livestock emissions) and is produced by methanogenic archaea in the rumen (Wang *et al*., 2024). Nitrous oxide (N_2O) is the second most important GHG emitted by livestock production, mainly derived from urine and dung deposited on pastures due to high nitrogen (N) excretion (Pacheco *et al*., 2008). In temperate, intensively managed pastures, high N content and moderate energy values can lead to excess ammonia (NH_3) production in the rumen, potentially exceeding the microbial assimilation capacity and resulting in increased urea formation and urinary N excretion (Beltran *et al*., 2019).

Therefore, nutritional strategies that simultaneously reduce ruminal CH_4 and NH_3 production are crucial for mitigating the environmental impact of grazing systems. Among these strategies, marine macroalgae have gained attention as dietary supplements or feed additives, depending on their inclusion level and mode of action. Within this group, red sea-

weeds, such as *Asparagopsis spp*., have been identified as potent CH_4 inhibitors because of their high bromoform content, which disrupts methanogenesis (De Bhowmick and Hayes, 2023). Although *Asparagopsis armata* has been reported as an invasive species in northern Chile (Villaseñor-Parada *et al*., 2018), *in vitro* studies on methane mitigation with *Asparagopsis armata* have been conducted in Chile, as described by Ungerfeld *et al*. (2025). However, its distribution is limited, and it is not currently considered a widely available or managed resource for livestock production systems in Chile. Beyond red seaweeds, other macroalgal taxa have also been investigated for their potential to modulate rumen fermentation. Brown seaweeds are characterised by low levels of halogenated compounds but high concentrations of phlorotannins (PhT), which are phloroglucinol-based polyphenols grouped into four structural classes (Ferreres *et al*., 2012). These compounds can form complexes with proteins and carbohydrates, thereby reducing protein degradation and NH_3 formation in the rumen (Manoni *et al*., 2024). In addition, PhT may indirectly reduce CH_4 production by decreasing fibre degradation and H_2 availability for methanogenesis (Kim *et al*., 2022). *In vitro* studies using *Laminaria digitata*, *Pelvetia canaliculata*, and *Alaria esculenta* have reported CH_4 reductions of approximately 38%, 51%, and 3-13%, respectively (de la Moneda *et al*., 2019; Ramin *et al*., 2019; Vissers *et al*., 2018).

Chile is the main producer of macroalgae in Latin America, with diverse and commercially valuable species present. The red alga *Gracilaria chilensis* is widely cultivated for its polysaccharides, particularly agar, a gelling compound extensively used in the food and pharmaceutical industries, whereas the brown seaweeds *Macrocystis pyrifera* and *Lessonia spicata* exhibit high cultivation potential (Camus *et al*., 2019). PhT content varies notably across species and harvest seasons. In Chile, Olate-Gallegos *et al*. (2019) reported higher PhT concentrations in *Macrocystis spp*. compared to *L. spicata*, and Tala *et al*. (2016) observed greater PhT levels in *M. pyrifera* during summer than in winter. Collectively, these findings suggest that *M. pyrifera* and *L. spicata* could contribute to the mitigation of CH_4 and N_2O emissions in pasture-based dairy systems in southern Chile. However, identifying the most effective species, inclusion rate, and optimal harvest period remains essential to maximise production efficiency.

A recent review highlighted the growing interest in using macroalgae, including Chilean brown seaweeds such as *M. pyrifera* and *Lessonia spp*., as potential strategies for mitigating enteric CH_4 emissions, although the results remain variable and context-dependent (Ungerfeld *et al*., 2025). Robles-Jimenez *et al*. (2024) evaluated several macroalgae species, including *M. pyrifera*, *Lessonia flavicans*, *Gigartina skottsbergii*, and *Ulva Lactuca*, collected in Chile, using an *in vitro* system based on an alfalfa hay substrate. Their results showed a reduction in CH_4 production, although the responses differed among species and were associated with changes in degradability and fermentation characteristics. These findings highlight that the response to macroalgae supplementation is species-specific, dose-dependent, and influenced by the dietary context. However, studies evaluating these seaweeds using substrates representative of temperate pasture-based systems and focusing on ruminal fermentation responses remain scarce. Because additive efficacy depends on diet composition (Durmic *et al*., 2025), evaluating these species under different forage substrates is necessary to better understand their effects on ruminal CH_4 and NH_3 production. This study aimed to evaluate the effects of Chilean brown and red seaweeds on *in vitro* rumen fermentation, methane production, and bacterial community structure using a pasture-based substrate.

MATERIALS AND METHODS

Experimental design and treatments

Seven treatments were evaluated using an *in vitro* ruminal fermentation system in a randomised block arrangement, including three seaweed species (*Lessonia spicata* [Ls], *Macrocystis pyrifera* [Mp], and *Gracilaria chilensis* [GCh]) collected in two seasons (winter: September 2021; summer: December 2021), and a control treatment without algae inclusion. The control diet consisted of 80% freeze-dried permanent pasture (dominated by *Lolium perenne*: 15.9% CP, 41.8% NDF, 28.0% ADF, 2.74 Mcal ME kg DM^{-1}, and 8.8% ash) and 20% grain-based concentrate (14.5% CP, 27.3% NDF, 9.5% ADF, 2.97 Mcal ME kg DM^{-1}, and 5.3% ash), simulating a typical diet for grazing dairy cows in southern Chile. Seaweeds were included at 10% of the dry matter (DM) diet, replacing the concentrate, following the inclusion levels used in a previous *in vitro* screening study (Mora *et al*., 2025). Two samples of each seaweed (per season), pasture, and concentrate were analysed before incubation and used to formulate the experimental diet. According to the chemical composition of seaweed, as detailed in Table 1, all diets were formulated to be isoenergetic and isoproteic.

Each treatment was performed in triplicate (technical replicates) within each fermentation run (biological replicates), with a total of three independent runs conducted per treatment, following the experimental approach described by Mora *et al*. (2025).

Feed collection and analysis

Seaweeds were manually collected during winter and summer of 2021 from the coast of Los Lagos Region, Chile (41°47′ S, 72°27′ W). Whole thalli (30 kg fresh weight per species) were harvested, excluding the holdfast in brown algae, and rinsed with seawater to remove sand and epiphytes. Samples were transported to the laboratory on ice, cut into 3-4 cm pieces, and frozen at -80 °C. Pasture and concentrate were collected in spring 2021 from the INIA Remehue Experimental Farm (40°31'S, 73°03'W; Osorno, Chile) and frozen at -80 °C. Seaweed, pasture, and concentrate were freeze-dried prior to incubation.

For laboratory analysis, all samples were freeze-dried,

Table 1.
Chemical composition of treatments (dry weight basis) with seaweeds included at 10% of dietary dry matter.

	Control	Winter			SEM	Summer			SEM
		GCh	Ls	Mp		GCh	Ls	Mp	
Crude protein (% DM)	15.6	16.4	15.5	15.3	0.28	15.4	15.5	15.4	0.04
Neutral detergent fiber (% DM)	38.9	39.5	37.8	37.8	0.49	39.1	37.6	37.1	0.59
Acid detergent fiber (% DM)	24.3	24.2	24.5	24.4	0.09	24.3	24.3	24.1	0.08
Metabolizable energy (Mcal kg^{-1} DM)	2.79	2.69	2.70	2.68	0.03	2.70	2.72	2.70	0.01
Ether extract (% DM)	2.8	2.7	2.8	2.7	0.02	2.7	2.7	2.7	0.01
Ash (% DM)	8.1	10.3	10.7	11.4	0.81	11.1	10.2	11.1	0.29

DM: Dry matter; GCh: *G. chilensis*, Ls: *L. spicata*, Mp: *M. pyrifera*; SEM: Standard error of mean.

ground (1 mm), and analysed for DM, crude protein (CP), ether extract (EE), and acid detergent fibre (ADF) (AOAC, 1996), and neutral detergent fibre (NDF) (Van Soest *et al*., 1991). Metabolisable energy (ME) was estimated by regression from digestible organic matter in the DM, which was determined *in vitro* according to Tilley and Terry (1963), following the approach of Garrido *et al*. (1981). The phlorotannin content (phloroglucinol equivalents; [PGE]) in brown seaweeds was determined using the Folin-Ciocalteu assay (Leyton *et al*., 2016).

In vitro incubations

In vitro fermentation was conducted as described by Mora *et al*. (2025). Rumen fluid was obtained from three rumen-cannulated Holstein-Friesian cows (495 ± 8.5 kg body weight; 16.2 ± 1.5 kg milk d^{-1}; 240 ± 35 days in milk) fed a 70:20 pasture-to-concentrate diet. Approximately 0.5 L of rumen content was collected from the cranial, caudal, and ventral sacs before the morning meal (45 min before incubation). Samples from each cow were squeezed, pooled in equal proportions, and transported to the laboratory in pre-warmed thermos flasks. The pooled content was filtered using a polyester filter cloth under O_2-free CO_2 at 39 °C, allowing protozoa and fine particles with adherent bacteria to pass through and be incorporated into the inoculum used for the *in vitro* fermentation assays. Then, 10 mL of inoculum was added to 160 mL bottles with 85 mL of Goering-Van Soest medium (Goering & Van Soest, 1970), 4 mL of reducing solution (0.625 g of Cysteine-HCl in 95 ml ultra-pure H_2O, 4 ml NaOH 1M, and 0.625 g of sodium sulphide), and 1 g DM of substrate. The bottles were then incubated anaerobically at 39 °C in a shaking water bath (50 rpm) for 48 h.

For each fermentation run (n = 3), a total of 37 bottles were incubated to evaluate all treatments. Two blank bottles without substrate were included to estimate the net production of fermentation end-products. Due to the maximum capacity of the incubation system, each treatment was incubated in triplicate for 48 h (n = 3), which was considered the main endpoint for fermentation and CH_4 production. Additionally, duplicate bottles were incubated for 4 h (n = 2) to assess early fermentation dynamics, considering that temperate pastures in the vegetative stage exhibit rapid degradation rates. This approach allowed all treatments to be evaluated within each run while maintaining sufficient replication across the three independent incubation runs.

Sampling and analysis

Gas pressure was measured at 2, 4, 6, 8, 10, 12, 14, 24, 36, and 48 h using a pressure transducer (PCE Instruments, Spain). Gas samples (10 mL) were collected at 4, 24, and 48 h and stored in pre-evacuated tubes (Labco Ltd., Lampeter, UK) for CH_4 determination by gas chromatography (Perkin Elmer Clarus 580, USA). The system was fitted with a 60/80 Carboxen 1000 packed column (Supelco, Bellefonte, PA, USA) and a thermal conductivity detector (TCD). Analyses were conducted under isothermal conditions at 180 °C using nitrogen (N2) as the carrier gas at a flow rate of 30 mL min^{-1}. After gas sampling, the bottles were shaken to homogenise the medium. The gas produced at each sampling time was corrected by subtracting the gas volume from blanks. Cumulative gas production data were fitted to the generalised Michaelis-Menten model without a lag phase, as described by France *et al*. (2007). CH_4 production at 4, 24, and 48 h was calculated according to the methodology proposed by Tavendale *et al*. (2005).

After 4 and 48 h of incubation, fluids were pooled by treatment, pH was measured (Orion Star A214, Thermo Fisher Scientific, Waltham, MA, USA), and 10 mL aliquots of the pooled fermentation fluid were acidified with 0.2 mL sulfuric acid (50% w w^{-1}) and frozen at -20 °C for NH_3 and short-chain fatty acid (SCFA) analysis by colorimetry (Kaplan, 1969) and gas chromatography (Beltran *et al*., 2019),

respectively. After 48 h, subsamples (1 mL) were stored at -80 °C for DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis. The residual material was centrifuged (32,000 x g, 10 min, 4 °C), freeze-dried, and analysed for DM and N (AOAC, 1996) to estimate apparent digestibility.

PCR and denaturing gradient gel electrophoresis analysis (DGGE)

Rumen bacterial community analysis was conducted using liquid samples collected after 48 h of *in vitro* incubation. Total microbial genomic DNA (gDNA) was extracted and standardised to a concentration of 20 ng μl^{-1} using the repeated bead-beating method described by Yu *et al*. (2005). The gDNA concentration and purity were determined using a Maestrogen spectrophotometre (Maestrogen, Hsinchu City, Taiwan). Bacterial PCR-DGGE analysis targeted the V3 region of the 16S rRNA gene, which was amplified using the primers 341f-GC and 543r (Klieve *et al*., 2007; Muyzer *et al*., 1993). Amplification and DGGE procedures were performed as described by Martínez *et al*. (2012). Purified PCR products were quantified using a NanoQuant Plate (Tecan Group Ltd., Männedorf, Switzerland) according to the manufacturer's recommendations. Seven composite samples corresponding to all treatments (control group plus six seaweed treatments), each obtained by pooling three replicates per treatment, were subjected to DGGE. A total of 100 ng of each purified PCR product was loaded per well on an 8% polyacrylamide gel with a 30-60% urea/formamide gradient in 0.5X TAE buffer. Gels were electrophoresed at 60 °C and 100 V for 18 h using the DCode System (Bio-Rad, Hercules, CA, USA), normalised with a multiband ladder, and visualised by silver staining (Kocherginskaya *et al*., 2001).

Statistical analysis

All statistical analyses were conducted in R (R version 4.3.2; R Core Team, Vienna, Austria) using RStudio (version 2026.1.1). Linear mixed-effects models were fitted using the lme4 package, and the estimated marginal means and planned contrasts were obtained using the emmeans package. The model assumptions of normality and homoscedasticity were evaluated through visual inspection of residual diagnostics, including residuals vs. fitted values plots and normal Q-Q plots. No data transformation was required.

To evaluate seasonal effects, an initial linear mixed-effects model including treatment seaweed, harvest season (for seaweed treatments only), and their interaction was fitted, with incubation run (n = 3) included as a random effect. Because the control treatment was not associated with a harvest season, the interaction between season and treatment was evaluated only within the seaweed treatments. Models with and without the interaction term were compared using likelihood ratio tests and the Akaike Information Criterion (AIC). Because the interaction term did not significantly improve the model fit, it was not retained in the final model.

Therefore, the final model included seaweed and seasonal effects through their combined factors, with block as a random effect. The estimated marginal means were obtained using the emmeans package.

Planned contrasts were specified a priori to address the main biological hypotheses: CTR-SW (control treatment vs. seaweed treatments averaged across seasons), LS-MP (comparison between the two brown seaweeds, *L. spicata* and *M. pyrifera*), GCH-MP (comparison between the red seaweed *G. chilensis* and the brown seaweed *M. pyrifera*), and WIN-SUM (winter vs. summer harvest seasons within seaweed treatments). Sentence-case abbreviations (e.g., Mp, Ls, and GCh) were used throughout the manuscript to refer to individual seaweed species, whereas uppercase abbreviations were used to denote planned contrasts between treatments.

Because these contrasts were defined a priori based on biological hypotheses, no additional adjustments for multiple comparisons were applied. Statistical significance was declared at $P \leq 0.05$, and tendencies were discussed when $0.05 < P \leq 0.10$.

Bacterial community composition was assessed using dendrogram analysis and biodiversity indices. DGGE profiles were digitised, and Pearson similarity matrices and UPGMA dendrograms (2% optimisation) were generated using Cliqs 1D (TotalLab Ltd., Newcastle Upon Tyne, UK). Alpha diversity indices were calculated from band intensity matrices, including species richness (S, number of bands per well), Shannon-Weiner index ($H' = -\Sigma$ [Pi ln Pi], Pi = relative band intensity), and evenness ($E = H'/\ln S$).

RESULTS

Seaweed chemical composition

Chemical composition of seaweed (Figure 1) was modified by species ($P < 0.05$), with the exception of EE ($P > 0.05$). It was also observed an interaction between species and season ($P < 0.05$) for CP, ADF, ME, DMD, ash, P, K and PhT. During winter, CP and NDF were greater and ADF, ash and non-fibrous carbohydrates (NFC) were lower for GCh compared to other seaweeds, respectively, while PhT content were 88% greater for Ls compared to Mp. During summer, CP was greater for Ls compared to Mp, while ME and d-value were greater for Ls compared to GCh. The content of NDF and ADF were greater and lower for GCh and Mp compared with Ls, respectively. Ash content was lower for Ls. Phlorotannin content was similar among brown seaweeds (Mp and Ls), averaging 3.07 mg PGE/kg DM.

Table 2.
Effect of seaweed inclusion in a pasture-based diet on *in vitro* production of ammonia and short chain fatty acids.

		Winter			Summer				P value				
	Control	GCh	Ls	Mp	GCh	Ls	Mp	SEM	Trt	CTR-SW	LS-MP	GCH-MP	WIN-SUM
4 h of incubation													
Ammonia (mmol L^{-1})	12.0	11.7	11.9	11.3	11.8	12.0	11.9	0.36	0.36	0.27	0.15	0.89	0.17
Total SCFA (mmol L^{-1})	37.5	32.9	33.9	28.5	35.5	35.2	27.9	4.05	0.04	< 0.01	< 0.01	0.05	0.42
Acetate (%)	64.4	65.3	64.4	65.8	64.5	64.2	65.5	1.37	0.35	0.22	0.02	0.28	0.37
Butyrate (%)	11.1	10.5	10.7	9.1	10.8	10.9	8.9	0.71	< 0.01	< 0.01	< 0.01	< 0.01	0.73
Propionate (%)	23.2	22.9	23.5	23.8	23.4	23.6	24.3	0.94	0.05	0.08	0.07	0.01	0.10
Other SCFA (%)	1.34	1.3	1.4	1.2	1.3	1.3	1.3	0.43	0.77	0.47	0.08	0.23	0.92
A:P ratio	2.79	2.9	2.7	2.8	2.8	2.7	2.7	0.16	0.56	0.59	0.85	0.36	0.14
48 h of incubation													
Ammonia (mmol L^{-1})	24.4	24.8	27.7	21.5	23.6	24.1	20.4	0.51	< 0.01	0.01	< 0.01	< 0.01	0.08
Total SCFA (mmol L^{-1})	85.4	81.8	84.5	86.9	83.0	85.0	78.0	6.85	0.93	0.58	0.64	0.89	0.62
Acetate (%)	61.1	62.3	61.7	59.8	61.7	61.1	59.3	1.04	0.13	0.75	0.01	< 0.01	0.40
Butyrate (%)	13.3	12.5	12.5	12.2	12.6	12.7	12.5	0.65	0.79	0.02	0.50	0.89	0.69
Propionate (%)	22.9	22.4	23.1	25.2	23.0	23.6	25.6	0.69	< 0.01	< 0.01	< 0.01	< 0.01	0.09
Other SCFA (%)	2.67	2.80	2.67	2.81	2.71	2.70	2.64	0.22	0.98	0.68	0.79	0.76	0.61
A:P ratio	2.68	2.79	2.68	2.38	2.69	2.60	2.32	0.11	< 0.01	0.05	< 0.01	< 0.01	0.16

A:P: acetate-to-propionate ratio; CTR-SW: control treatment vs. seaweed contrast; GCh: G. chilensis; GCH-MP: GCh vs. *Mp contrast*; Ls: *L. spicata*; LS-MP: Ls vs. Mp contrast; Mp: M. pyrifera; SCFA: short-chain fatty acids; SEM: standard error of the mean; Trt: overall treatment effect; WIN-SUM: winter vs. summer contrast. Specific SCFA are expressed as a proportion of the total SCFA (%).

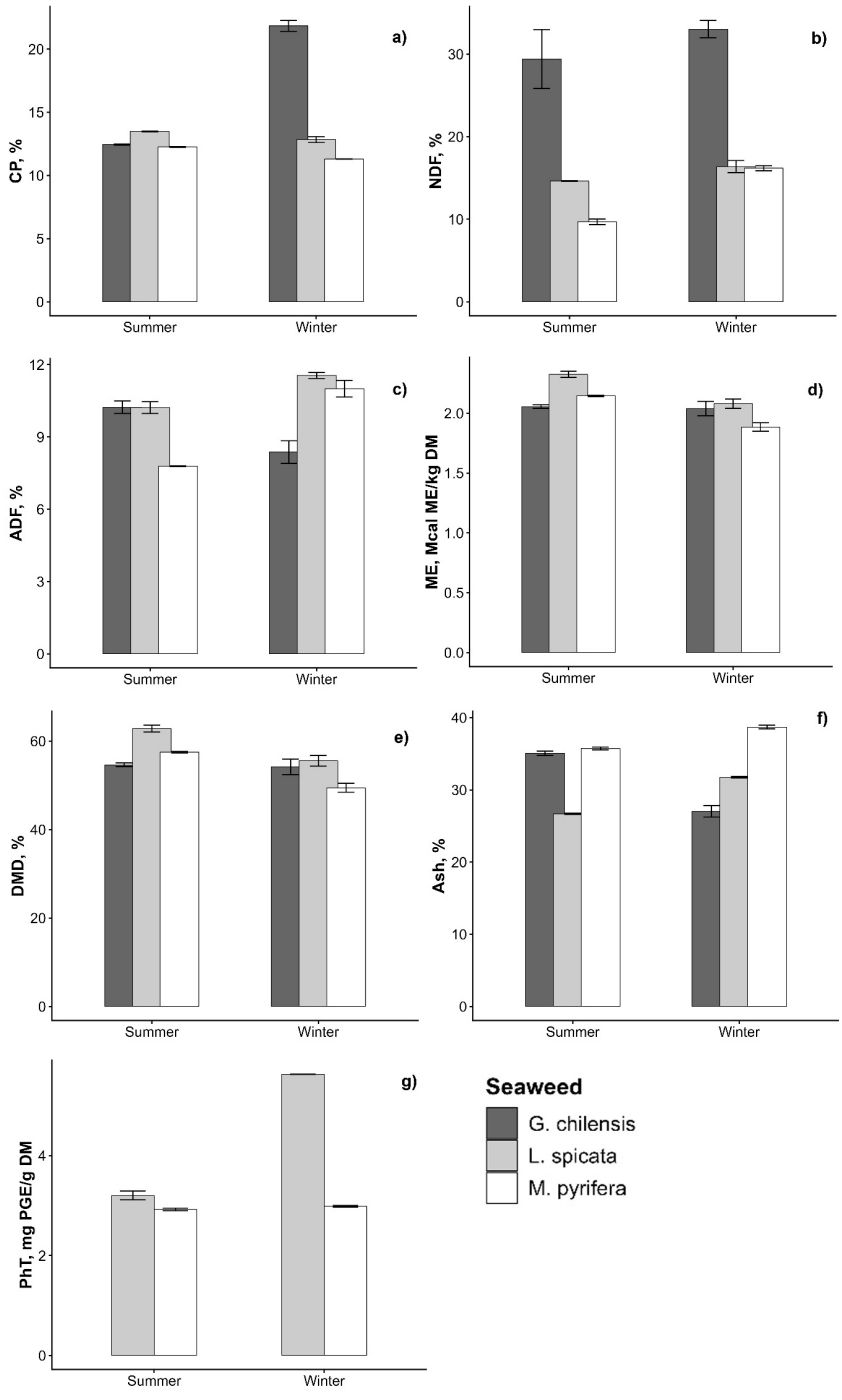

Figure 1.
Seasonal variation (Summer and Winter) in the concentration (based on dry matter) of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), metabolizable energy (ME), dry matter digestibility (DMD), ash, and Phlorotannin (PhT: PGE: phloroglucinol equivalents) for *M. pyrifera*, *L. spicata* and *G. chilensis*. Error bars represent the standard error of the mean (SEM). Phlorotannins were measured only in the brown seaweeds (*M. pyrifera* and *L. spicata*), as they are characteristic compounds of brown algae.

Ammonia and short-chain fatty acid

The effects of the treatments on *in vitro* NH_3 and SCFA are shown in Table 2. After 4 h of incubation, ruminal NH_3 was not modified by seaweed and harvest (CTR-SW: $P = 0.27$; LS-MP: $P = 0.15$; GCH-MP: $P = 0.89$; WIN-SUM: $P = 0.17$). After 48 h of incubation, control treatment showed a 3% higher NH_3 concentration compared to seaweed treatments (CTR-SW; $P = 0.01$). Lp showed a 19% higher NH_3 concentration compared to Mp (LS-MP; $P < 0.01$), whereas GCh increased by 13.4% rumen NH_3 compared to Mp (GCH-MP; $P < 0.01$). Season did not modify ruminal NH_3 concentration (WIN-SUM; $P = 0.08$).

After 4 h of incubation, the control treatment showed a higher total SCFA and butyrate proportion compared with seaweed treatments (CTR-SW, $P < 0.01$). When brown seaweeds were compared, Ls showed a higher total SCFA and butyrate proportion, but a lower acetate proportion compared to Mp (LS-MP; $P < 0.01$). The proportion of propionate was greater for Mp compared to GCh (GCH-MP; $P = 0.01$), whereas butyrate proportion was higher in GCh, increasing by 15.4% compared to Mp (GCH-MP; $P < 0.01$). After 48 h, acetate was 2.5% lower for Mp than Ls (LS-MP; $P = 0.01$) and GCh (GCH-MP; $P < 0.01$), whereas butyrate was 6% greater for control treatment than seaweed treatments (CTR-SW; $P = 0.02$). Seaweed treatments increased the propionate molar percentage by 11% relative to control treatment (CTR-SW; $P < 0.01$). Among seaweed species, Mp showed a greater proportion of propionate than Ls and GCh (LS-MP and GCH-MP; $P < 0.01$), reducing the acetate:propionate (A:P) ratio by 12.9% compared with both species.

Rumen digestibility

The *in vitro* ruminal digestibility of DM, OM, and N is presented in Table 3. Organic matter and N digestibility were 6.6% and 7.2% greater for control treatment compared to seaweed treatments, respectively (CTR-SW; $P = 0.03$ and $P = 0.02$, respectively). When brown seaweeds were compared (LS-MP), DM ($P = 0.03$), OM ($P = 0.03$), and N digestibility ($P < 0.01$) were 5.1%, 10%, and 7.4% lower for Mp compared to Ls, respectively. The harvest season had no effect on DM, OM, and N ruminal digestibility (WIN-SUM; $P = 0.88$, $P = 0.87$, and $P = 0.55$, respectively).

Table 3.
In vitro ruminal digestibility of dry matter (DM), organic matter (OM), and nitrogen (N) in pasture-based diet including brown and red seaweeds after 48 h of incubation.

		Winter			Summer				P value				
	Control	GCh	Ls	Mp	GCh	Ls	Mp	SEM	Trt	CTR-SW	LS-MP	GCH-MP	WIN-SUM
DM (%)	84.9	82.3	81.9	80.3	80.9	84.0	80.0	2.18	0.68	0.07	0.03	0.78	0.88
OM (%)	83.6	79.7	73.9	77.6	78.2	81.6	77.2	1.97	0.41	0.03	0.03	0.99	0.87
N (%)	88.3	84.0	82.6	80.5	82.7	85.4	76.5	2.01	0.04	0.02	< 0.01	0.17	0.55

CTR-SW: control treatment vs. seaweed contrast; GCh: *G. chilensis*; GCH-MP: GCh vs. Mp contrast; Ls: *L. spicata*; LS-MP: Ls vs. Mp contrast; Mp: *M. pyrifera*; SEM: standard error of the mean; Trt: overall treatment effect; WIN-SUM: winter vs. summer contrast.

Gas production and methane

The effects of algae species and harvesting season on gas and CH_4 production are presented in Table 4 and Figure 2. Gas production (mL g OM^{-1}) was similar between control treatment and seaweed treatments (CTR-SW) at 4 h ($P = 0.44$), 24 h ($P = 0.86$) and 48 h ($P = 0.84$); however, it was lower for Mp compared to Ls (LS-MP; $P < 0.01$) and GCh (GCH-MP; $P < 0.01$) at 4, 24, and 48 h of incubation. CH_4 concentration (expressed as a percentage of total gas production) was reduced for seaweed treatments compared to the control treatment (CRT-SW) at 4 ($P = 0.02$), 24 ($P < 0.01$) and 48 h ($P < 0.01$). In particular, CH_4 concentration was 18.7% and 13.4% lower for Mp compared to Ls at 4 (LS-MP; $P = 0.03$) and 24 h (LS-MP; $P = 0.01$), respectively, and 20% lower compared to GCh at 4 h (GCH-MP; $P < 0.05$). Similarly, CH_4 production (mL g OM^{-1}) was lower for seaweed compared to control treatment at 24 and 48 h of incubation (CTR-SW; $P < 0.01$), with Mp showing the greatest reduction among algae species, especially at 4 and 24 h of incubation ($P = 0.04$). A tendency to lower CH_4 production was also observed at 48 h for Mp compared to Ls (LS-MP; $P = 0.08$) and GCh (GCH-MP; $P = 0.09$).

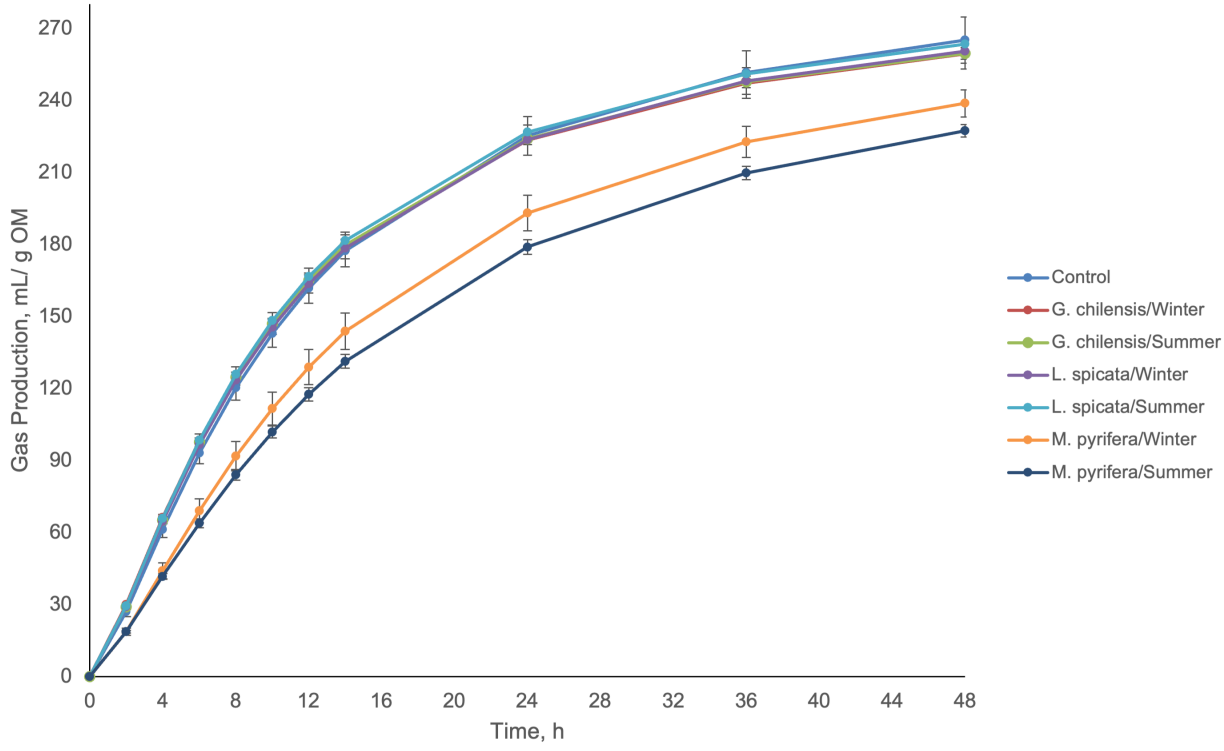

Figure 2.
In vitro gas production (mean ± standard error of mean) for pasture-based diet including brown and red seaweeds (10% of inclusion) harvested in summer and winter in southern Chile.

Table 4.
Effect of algae species and harvesting season on *in vitro* gas and methane production in a pasture-based diet.

		Winter			Summer				*P* value				
	Control	GCh	Ls	Mp	GCh	Ls	Mp	SEM	Trt	CTR-SW	LS-MP	GCH-MP	WIN-SUM
Gas production (mL g OM^{-1})													
4 h	61	69	68	55	68	69	54	4.59	0.10	0.44	< 0.01	< 0.01	0.84
24 h	225	234	234	212	233	237	204	7.74	0.05	0.86	< 0.01	< 0.01	0.74
48 h	265	272	273	254	270	276	249	5.56	0.07	0.84	< 0.01	< 0.01	0.77
Methane (%)													
4 h	8.5	8.4	7.7	6.3	7.2	7.6	6.2	0.55	0.13	0.02	0.03	0.04	0.53
24 h	15.9	13.8	14.5	13.1	13.3	14.7	12.2	0.88	0.10	< 0.01	0.01	0.42	0.82
48 h	15.8	14	13.5	13.1	14	12.8	13.5	0.58	0.02	< 0.01	0.75	0.49	0.81
Methane (mL g OM^{-1})													
4 h	5.8	6.8	6.1	4.1	5.6	6.1	3.7	0.50	< 0.01	0.18	< 0.01	< 0.01	0.17
24 h	40.8	37.7	39.2	32.2	35.9	40.5	28.1	2.01	< 0.01	< 0.01	< 0.01	0.02	0.39
48 h	47.5	44.2	42.6	38.5	43.8	41.1	38.7	2.22	0.07	< 0.01	0.08	0.09	0.73

CTR-SW: control treatment vs. seaweed contrast; GCh: *G. chilensis*; GCH-MP: *GCh* vs. *Mp* contrast; Ls: *L. spicata*; LS-MP: *Ls* vs. *Mp* contrast; Mp: *M. pyrifera*; SEM: standard error of the mean; Trt: overall treatment effect; WIN-SUM: winter vs. summer contrast. OM: organic matter.

DGGE profiles of rumen bacterial diversity

There was variation among the treatments in the DGGE patterns of the bands (Figure 3). It is possible to describe a similar band pattern including GCh (winter and summer), Mp (summer), and control treatment, clustering together in the DGGE profile. The DGGE bacterial profile of treatment Mp (winter) differed from the principal cluster by showing a less intense pattern band profile. The Ls treatments harvested in both winter and summer also clustered together, forming a distinct group with a different banding pattern compared with the other treatments.

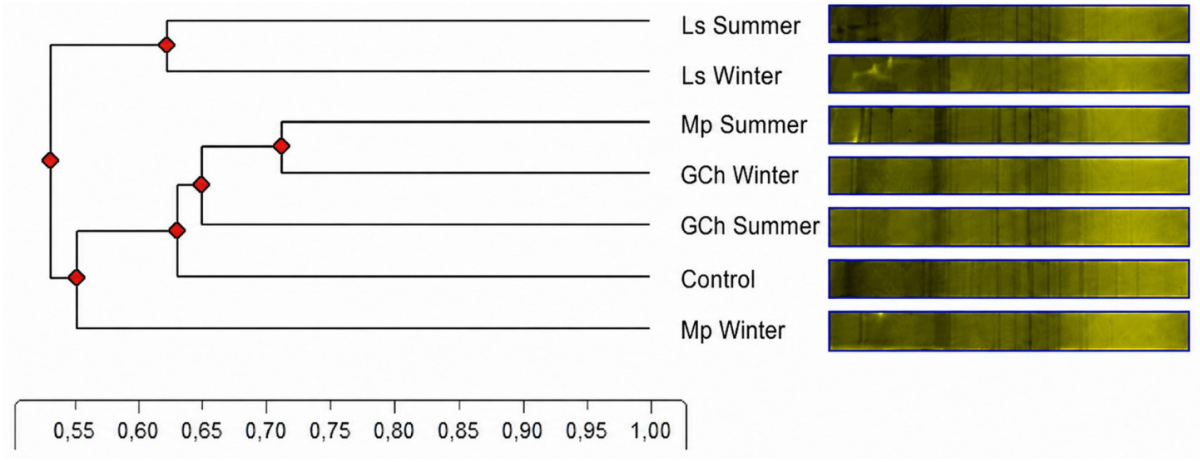

Figure 3.
Dendrogram of rumen bacterial denaturing gradient gel electrophoresis (DGGE) profile of the rumen *in vitro* batch cultures using the Pearson similarity matrix. GCh: *G. chilensis*, Ls: *L. spicata*, Mp: *M. pyrifera*.

Table 5.
Alpha diversity indices of bacterial communities based on denaturing gel gradient electrophoresis profile.

		GCh		Ls		Mp	
Alpha Diversity Indices	**Control**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**
Species richness (S)	33	32	32	21	24	27	27
Shannon Weiner (H')	3.11	3.25	3.07	2.81	2.71	2.76	2.76
Evenness (E)	0.89	0.94	0.88	0.92	0.85	0.84	0.84

GCh: *G. chilensis*, Ls: *L. spicata*, Mp: *M. pyrifera*.

Alpha diversity indices based on DGGE rumen bacterial profiles allowed a general initial description of bacterial communities (Table 5). In the case of GCh in winter or summer, their species richness (S) and Shannon (H') indices were higher than those of the other treatments, and their distribution (E) was proportional among the treatments. Treatments including Ls in winter and summer showed fewer bands and a lower Shannon index than the other treatments. However, it still had a highly diverse community with homogeneous distribution (E).

DISCUSSION

This study evaluated the effects of three macroalgae harvested in two seasons (winter and summer) on *in vitro* fermentation parameters, CH_4 production, and bacterial abundance using a relatively high inclusion rate (10% DM basis).

Ammonia and volatile fatty acid

The reduction in NH_3 with Mp inclusion may be associated with its PhT content, which may limit ruminal N digestibility through hydrogen bonding with proteins and fibre (Kim *et al*., 2022). This aligns with the lower N digestibility observed for the CTR-SW and LS-MP contrasts. Although Mp contained less total PhT than Ls (2.96 ± 0.04 and 4.41 ± 1.40 mg PGE g DM^{-1}, respectively), the differences in NH_3 reduction among species suggest that factors beyond total PhT concentration may be involved. Similar effects have been observed in other brown seaweeds, including *Sargassum horneri* (Park *et al*., 2022) and *Laminaria digitata* (Vissers *et al*., 2018).

The absence of an effect on ruminal NH_3 at 4 h suggests a delay in fermentation, likely due to alginate, a viscous polysaccharide abundant in brown seaweed cell walls (Stiger-Pouvreau *et al*., 2016), which may limit microbial access to substrates, as alginate matrices can protect compounds and enable their gradual release under gastrointestinal conditions (Almassri *et al*., 2025). Although total SCFA was reduced at 4 h, it recovered at 48 h, supporting a delayed fermentation pattern, as previously reported for brown seaweeds (Véliz *et al*., 2023).

Mp increased the propionate proportion at both incubation times, reducing the acetate: propionate (A:P) ratio, a pattern typical of CH_4 inhibitors owing to hydrogen (H_2) redirection toward propionate formation (Kim *et al*., 2022). Similar results were reported for *Ecklonia radiata* (Mihaila *et al*., 2022) and PhT extracts (Kim *et al*., 2022).

Gas production and methane

Mp was the only seaweed species that reduced total gas production at 24 and 48 h, consistent with the reduced OM digestibility (de la Moneda *et al*., 2019). Lower DM and OM digestibility for Mp compared to control treatment (CTR-SW) and Ls (LS-MP) likely reflects tannin-nutrient complex formation, limiting microbial access to fibre. Similar reductions in digestibility and total gas production have been observed in PhT-containing algae (Mihaila *et al*., 2022). Moreover, the relatively high ash content of Mp may also have reduced the proportion of fermentable OM, further contributing to the decline in gas and CH_4 production.

The inclusion of Mp decreased CH_4 (mL CH_4 g OM^{-1} incubated) by 16%, 22%, and 11% at 4, 24, and 48 h, respectively. This inhibition may result from three main mechanisms: 1) direct effects of PhT on methanogenic archaea and associated microbial groups (e.g. protozoa); 2) decreased H_2 availability due to reduced fibre digestion (Kim *et al*., 2022); and 3) a lower amount of organic matter available for fermentation. Although Ls had a higher PhT content, it did not decrease CH_4 production, suggesting that the structural characteristics of PhT or other associated compounds, rather than the total content, are key to explaining the differences among seaweeds. Kim *et al*. (2022) demonstrated that CH_4 inhibition is more closely related to the presence of hydroxyl groups and ether linkages within PhT structures than to their absolute concentration. Similarly, Pandey *et al*. (2022) found no direct relationship between total phenols and Methanobrevibacter abundance, suggesting that phlorotannin may affect other methanogenic groups or shift the archaeal community structure, thereby influencing methanogenesis through additional mechanisms.

Although Mp decreased the total gas production and CH_4, it also reduced the CH_4 proportion in the gas, indi-

cating its effects on both microbial metabolism and substrate utilisation.

In line with these results, previous *in vitro* studies using Chilean macroalgae have shown that species such as Mp and Lessonia spp. can reduce CH_4 production (mL CH_4 g^{-1} DM) under alfalfa hay-based diets (Robles-Jimenez *et al*., 2024).

Given that DGGE profiles showed some treatment-related clustering, but overall diversity indices remained relatively similar among treatments, the observed CH_4 reduction likely reflects specific effects on methanogenic archaea or their syntrophic partners, rather than broad shifts in the bacterial community.

DGGE profiles of rumen bacterial diversity

DGGE profiles revealed differences in dominant bacterial communities among the treatments. Samples from GCh (winter and summer), Mp (summer) and the control treatment clustered together, whereas Mp (winter) showed a weaker band pattern. In contrast, Ls (both seasons) formed an independent cluster with fewer bands, suggesting a different bacterial profile. The separation of Ls likely reflects the structural characteristics of its PhT, which may selectively affect certain bacterial groups.

No clear reduction in bacterial diversity was observed across the treatments based on the DGGE profiles. Likewise, no differences in diversity indices were found between seasons, suggesting that seasonal variation did not markedly affect the dominant bacterial populations. However, DGGE only detects the most abundant taxa (McSweeney *et al*., 2009). Thus, minor shifts in the community cannot be excluded. In addition, it should be noted that microbial DNA was extracted from the liquid phase of the incubation, which may not fully represent the rumen microbiota, as a large proportion of microorganisms are associated with solid particles. Therefore, potential shifts in particle-associated microbial populations may have been underestimated in this study. Further 16S rRNA sequencing of bacterial and archaeal communities is needed to improve our understanding of the mechanisms driving CH_4 reduction in Mp.

Implications of the study

No significant effects of harvesting season (winter or summer) or its interaction with algal species were observed on ruminal NH_3, SCFA, or CH_4 yields. This suggests that seaweed can be harvested throughout the Chilean production season (November-May) without affecting its ruminal impact. Among the three species evaluated, only Mp produced a moderate reduction in CH_4 production and NH_3 concentration with a forage-based substrate; however, these effects were accompanied by reduced digestibility, limiting its applicability as a CH_4 mitigation strategy under the conditions evaluated in this study.

The reduction in ruminal NH_3 may indicate shifts in N partitioning associated with decreased ruminal protein degradation. Similar relationships were previously reported by Beltran *et al*. (2019), who observed an association between lower ruminal NH_3 concentrations and reduced urinary N losses in grazing dairy cows. This is environmentally relevant because urinary N represents a major source of N_2O emissions in grazing systems, with urine patches emitting approximately five times more N_2O than dung per unit of excreted N (van der Weerden *et al*., 2011). Nevertheless, the reductions in NH_3 observed in the present study occurred concurrently with decreased digestibility, representing an important drawback that may limit the practical utilisation of these nutritional strategies not only for methane mitigation but also for improving N-use efficiency under the evaluated conditions. Despite these limitations, studies such as the present one are essential to generate the scientific evidence required to better understand the trade-offs associated with these approaches and to support the development of feasible nutritional interventions capable of modulating ruminal N dynamics while maintaining adequate fermentation efficiency in cattle systems.

In addition, previous studies have suggested that Mp may influence meat quality traits, including a reduction in total lipid content, which is particularly relevant for consumers concerned about diet-health relationships. This could position Mp as a natural feed additive with the potential to contribute to the production of leaner meat products that better align with current consumer preferences (Jerez-Timaure *et al*., 2023). These findings suggest that the effects of this macroalga may be relevant in contexts other than CH_4 mitigation.

CONCLUSION

Macrocystis pyrifera showed the greatest potential to reduce CH_4 and NH_3 production *in vitro*, along with increased propionate production. However, these effects were accompanied by reduced nutrient digestibility, representing an important drawback that limits its practical application for both methane mitigation and N-use efficiency at the evaluated inclusion level. In contrast, *L. spicata* and *G. chilensis* showed limited mitigation potential. However, the secondary compounds present in these macroalgae may have additional biological effects that deserve further investigation. Overall, studies such as this are needed to better understand the trade-offs associated with macroalgae supplementation and support their practical use in ruminant systems.

Competing Interests Statement

The authors declare that they have no competing interests

Ethics Statement

Ethical approval for all procedures involving animals was obtained from the Animal Care and Use Committee of Instituto de Investigaciones Agropecuarias (Approval Certificate No. 02/2020).

Data availability statement

The datasets and model generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

Conceptualization, I.B. and J.P.K.; Methodology, I.B., J.P.K, A.B., S.P., E.M. and M.S.; Software: I.B.; formal analysis, I.B. and J.P.K.; investigation, I.B., J.P.K. and F.S.; data curation, I.B., J.P.K. and J.D.; writing—original draft preparation, I.B.; writing—review and editing, J.P.K., A.B., S.P., F.S., R.G.P., J.D. and E.V.; project administration, I.B.; funding acquisition: I.B. All authors have read and agreed to the published version of the manuscript

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