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Communication

Sub-Antarctic Macroalgae as Feed Ingredients for Sustainable Ruminant Production: In Vitro Total Gas and Methane Production

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Abstract: The sustainable meeting of the global quest for ruminant intensification dictates the need to identify alternative, eco-friendly, and safe feed ingredients. In this sense, macroalgae offer a new paradigm in sustainable ruminant feed supply. This study aimed to investigate the potential of sub-Antarctic macroalgae, including *Lessonia flavicans*, *Macrocystis pyrifera*, *Gigartina skottbergii*, and *Ulva Lactuca*, regarding their chemical composition, in vitro gas production, and CH_4 production. A completely randomized design consisted of a 96 h (h) incubation that included four different species and a control (alfalfa hay) with buffered rumen fluid. In vitro total gas, fermentation characteristics, and CH_4 production were evaluated. The highest and the lowest crude protein (CP) contents were for *U. lactuca* (185.9 g/kg) and *G. skottsbergi* (86 g/kg), respectively ($p < 0.0001$). All macroalage had lower levels of natural detergent fiber (NDF) and acid detergent fiber (ADF) compared to alfalfa hay ($p < 0.0001$). The highest potential of gas production (b) was for *M. pyriphera* (162.8 mL gas/g DM), followed by alfalfa (119.3 mL gas/g DM). However, *G. skottsbergi* and *M. pyriphera* showed the highest dry matter degradability at 96 h (68.49 and 67.62 mg/100 mg, respectively; $p < 0.0001$) and microbial crude protein (679.8 and 669.8 mg/g, respectively, $p < 0.0001$). All four tested algae produced lower amounts of methane compared to alfalfa hay ($p < 0.0001$). After 24 h of incubation, *M. pyriphera*, *L. flavicons*, *G. skottsbergi*, and *U. lactuca* reduced CH_4 by 99.7%, 98.6%, 92.9%, and 79.8%, respectively, when compared with the control. Also, all tested algae had lower ($p = 0.0001$) CH_4 production (ml CH_4/g Dry matter degradability, DMD) than alfalfa hay. The current results suggest that *M. pyriphera* and *L. flavicons* are promising feed additives for ruminants with eco-friendly production and acceptable CP content and DMD that could effectively mitigate CH_4 emissions. Overall, these findings suggest that macroalgae hold promise as a substitute feed source for sustaining ruminant production at the onset of global warming.

Keywords: greenhouse gas; macroalgae; methane; nutritive value; rumen fermentation; ruminant; sustainability

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1. Introduction

Ruminants will continue to play a crucial role in providing high-quality protein to feed the projected ~10 billion humans by 2050 [1]. However, the sustainability of ruminant systems has raised concerns since they contribute to ~41% of total agricultural greenhouse gas emissions (GHGE) and ~17% of the global anthropogenic enteric GHGE [2]. Globally,

ruminants are expected to emit about 80 to 95 million metric tons of CH_4 per year [3]. CH_4 generation also represents a substantial loss of energy for the host animal, often ranging from 2% to 12% of the total energy available [4]. Notably, it has been reported [3–5] that the enteric CH_4 emissions from ruminant production constitute the largest source of GHGs, emitting 46% of the CO_2 equivalent in dairy operations and 55% in small ruminant farming. Almost all the CH_4 generated in the rumen via methanogenis convert liberated hydrogen into CH_4, a by-product of the complex fermentative process in the rumen ecosystem. This system, rich in protozoa, bacteria, archaea, viruses, fungi, and bacteriophages, facilitates the conversion of ingested feed into energy and nutrients essential for the host ruminant [6].

After the United Nations [7] set the net zero emissions program and called for an urgent reduction in global emissions, strategies for mitigating CH_4 emissions became a top priority in ruminant research, especially via dietary approaches as an important management tool [8,9]. Some promising strategies for CH_4 mitigation include inhibiting methanogens, the defaunation of rumen protozoa, antibiotics, redirecting hydrogen from CH_4 production to other pathways, adjusting the dietary forage:concentrates ratio, and incorporating natural feed additives and phytocompounds [3,10]. It has been well documented that the ideal feed additives should reduce CH_4 emissions without deleterious effects on digestion efficiency or animal performance [10,11]. Recently, microalgae have been seen as one of the prospective feed alternative sources for sustainable-minded ruminant systems that is not only rich in vitamins, proteins, polysaccharides, and bioactive compounds but also reduces enteric CH_4 emissions [2,8,9]. Furthermore, macroalgae's superior growth rate, its enhanced biomass production, the feasibility of saltwater cultivation, and the absence of the need for arable land and industrial fertilizers are additional advantages over terrestrial plants [12].

Macroalgae, commonly known as seaweed, consist of a wide spectrum of 6000 to 10,000 marine species, populating the coastal zones across the globe, and they can be systematically classified into three primary categories based on their pigmentation: brown (phaeophyta), red (rhodophyta), and green (chlorophyta) [2]. Globally, the annual harvest of macroalgae reached ~36 million metric tons, with the market value being USD ~6 billion for various commercial applications [12]. Macroalgae species exhibit varied nutritional profiles; however, the majority boast high protein (25–40%), fat (10–30%), carbohydrate (5–30%), and neutral detergent fiber (NDF, 15.3–43.1%) contents, often matching or exceeding those in standard feeds like soybean meal, corn, and wheat [8,9,11]. Unlike terrestrial plants, the cell walls of seaweeds are primarily composed of alginates, with some cellulose, xylan, and xyloglucan [2]. It is well established that macroalgae, through the production of halogenated secondary metabolites such as bromoform, can significantly reduce CH_4 emissions by directly inhibiting methanogenesis [13]. Bromoform and other halogenated compounds can suppress methanogenesis and strongly reduce enteric CH_4 production by 0 to 98%, influenced by factors like the dosage, basal diet, and storage conditions [14]. Macroalgae application could also enhance feed efficiencies in ruminants by redirecting energy from the microbial methanogenesis pathway to more advantageous pathways for the animal, i.e., the production of volatile fatty acids (VFAs) [3,5,15]. *Lessonia flavicans* is a light brown to dark brown alga that is 60 cm to 4 m long, has a dichotomously divided thallus with long narrow laminar fronds, has a smooth surface, and does not have ribs. *Macrocystis pyrifera* has a yellow–brown thallus; its lanceolate laminae are unilaterally arranged and have pneumatocysts attached to the cylindrical stipe, up to 60 m long. *Gigartina skottbergii* is a red alga with small rhizoid-like excrescences at the base of the thallus, which allow it to adhere strongly to the substrate, with a length between 3 and 20 m. *Ulva lactuca* is a light green alga, with a smooth-edged expanded sheet-like rounded leaf that varies greatly in shape, with a length of up to 50 cm; all these algae are distributed in South America, South Africa, Australia, and New Zealand and subantarctic islands [16].

Here, we tested the potential of four different species of sub-Antarctic macroalgae from brown (*Lessonia flavicans* and *Macrocystis pyrifera*), red (*Gigartina skottbergii*), and green (*Ulva lactuca*) classes sourced from the Magallanes y de la Antartica Chilena Region, Chile,

for their chemical composition, in vitro gas yield, fermentation kinetics, and ability to mitigate enteric CH_4 production. We opted to analyze algae per se to avoid the complexity of dietary interactions (including chemical composition, physical form, particle size, etc.), which could affect results. This approach helps to clarify the isolated impact of algae, and by comparing it to high-quality forage, we aim to identify any limitations or successes when algae are introduced into mixed diets. Our hypothesis is that the administration of macroalgae affects in vitro fermentation and degradation, compared to alfalfa hay, and also reduces methanogenesis without negatively affecting rumen fermentation.

2. Results

Table 1 shows the chemical composition of selected macroalgae. The OM content of alfalfa hay was higher ($p < 0.0001$) than that of all tested macroalgae. The highest and the lowest CP concentrations were for *U. Lactuca* and *G. skottsbergi*, respectively ($p < 0.0001$). All macroalgae had lower levels of NDF and ADF compared to alfalfa hay ($p < 0.0001$). However, *L. flavicans* and alfalfa hay had similar ADL contents, higher than that of the other macroalgae ($p < 0.0001$).

Table 1. Chemical composition of different macro algae with potential use in ruminant diets.

Item	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [1]	*p*-Value
OM, g/kg	744.63 ^{b}	561.97 ^{e}	693.77 ^{c}	641.92 ^{d}	899.13 ^{a}	0.638	0.0001
CP, g/kg	86.00 ^{e}	141.55 ^{c}	111.86 ^{d}	185.91 ^{a}	154.50 ^{b}	1.054	0.0001
EE, g/kg	17.68 ^{a}	3.00 ^{d}	1.65 ^{e}	14.34 ^{b}	8.57 ^{c}	0.188	0.0001
NDF, g/kg	238.94 ^{c}	177.87 ^{c}	254.37 ^{b}	207.79 ^{c}	389.15 ^{a}	6.876	0.0001
ADF, g/kg	94.00 ^{c}	106.34 ^{b}	93.66 ^{c}	96.00 ^{c}	214.00 ^{a}	1.156	0.0001
ADL, g/kg	6.51 ^{b}	6.28 ^{b}	7.30 ^{a}	6.06 ^{b}	7.75 ^{a}	0.091	0.0001

Organic matter, OM; crude protein, CP; ether extract, EE; neutral detergent fiber, NDF; acid detergent fiber, ADF; acid detergent lignin, ADL; [1] Standard error of means. $^{a–e}$ Means within a row with different superscripts differ ($p \leq 0.05$).

The potential of gas production (b) was in the following order for *M. pyriphera* > alfalfa hay > *U. lactuca* > *L. flavicons* > *G. skottsbergi* ($p = 0.0013$, Table 2). While the highest ($p = 0.0001$) gas rate (c) was for alfalfa, followed by *L. flavicons*. Also, the highest and lowest lag time was for *L. flavicons* and *M. pyriphera*, respectively ($p < 0.0001$). The in vitro gas yield at all times (i.e., 6, 12, 24, 48, and 96 h) was higher ($p < 0.0001$) in the control (alfalfa hay), followed by that of *U. lactuca*. However, *G. skottsbergi* and *M. pyriphera* showed the highest dry matter degradability at 96 h (DMD96, $p < 0.0001$) and microbial crude protein production (MCP, $p < 0.0001$).

Table 2. In vitro rumen gas kinetics and fermentation profile of different macroalgae with potential use in ruminant diets.

Item [1]	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [2]	*p*-Value
b	29.73 ^{c}	162.82 ^{a}	50.95 ^{c}	102.33 ^{abc}	119.35 ^{ab}	16.519	0.0013
c	0.023 ^{c}	0.004 ^{d}	0.036 ^{b}	0.017 ^{c}	0.043 ^{a}	0.002	0.0001
Lag time	−0.617 ^{cd}	−1.821 ^{d}	4.311 ^{a}	−0.098 ^{c}	2.416 ^{b}	0.329	0.0001
Mean gas production in time (mL gas/g DM)							
6 h	4.44 ^{c}	7.78 ^{bc}	4.42 ^{c}	10.92 ^{b}	16.31 ^{a}	0.976	0.0001
12 h	6.82 ^{d}	12.45 ^{c}	10.87 ^{cd}	20.74 ^{b}	40.41 ^{a}	1.206	0.0001
24 h	11.55 ^{d}	14.68 ^{d}	25.13 ^{c}	33.42 ^{b}	73.66 ^{a}	1.621	0.0001
48 h	20.97 ^{d}	32.99 ^{cd}	41.18 ^{c}	58.56 ^{b}	102.28 ^{a}	3.142	0.0001
96 h	25.88 ^{d}	59.14 ^{c}	48.83 ^{c}	82.51 ^{b}	118.28 ^{a}	4.567	0.0001

Table 2. *Cont.*

Item [1]	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [2]	*p*-Value
DMD96	68.49 [a]	67.62 [a]	41.60 [b]	14.72 [c]	44.64 [b]	0.765	0.0001
ME	7.89 [e]	11.27 [c]	10.28 [d]	15.07 [b]	16.02 [a]	0.101	0.0001
MCP	679.80 [a]	669.81 [a]	404.98 [b]	132.48 [c]	413.95 [b]	7.226	0.0001
SCFA	0.05 [d]	0.06 [d]	0.10 [c]	0.14 [b]	0.32 [a]	0.007	0.0001
N-NH_3	26.91 [a]	21.05 [b]	21.47 [b]	30.67 [a]	31.0[7] [a]	2.293	0.0258

[1] b = potential cumulative gas production (mL/g DM), c = rate of gas production (h^{-1}), Lag time = initial lag for the onset of fermentation (h), Mean gas production in time = mL gas/g DM at different times, DMD96 = Dry matter degradability at 96 h (g/100 g), ME = Metabolizable energy (Mj/ kg DM), MCP = microbial crude protein (mg/g), SCFA = short chain fatty acids (mmol/200 mg), N-NH_3 = Ammonia N (mg/dl). [2] SEM: Standard error of pooled means. [a–e] Means within a row with different superscripts differ ($p \leq 0.05$).

Table 3 presents the in vitro methane production accumulated (mL CH_4/g DM) by macroalgae. All four tested algae produced lower amounts of methane after 3, 6, 9, 12, and 24 h of incubation compared to alfalfa hay ($p \leq 0.05$), with the numerically lowest values being for *M. pyriphera*, *L. flavicons*, and *G. skottsbergi.* After 24 h of incubation *M. pyriphera*, *L. flavicons*, *G. skottsbergi*, and *U. lactuca* reduced CH_4 by 99.7%, 98.6%, 92.9%, and 79.8%, respectively, when compared with alfalfa hay. Also, *M. pyriphera*, *L. flavicons*, and *G. skottsbergi* had lower ($p = 0.0001$) CH_4 production (mL CH_4/ g DMD) than alfalfa hay.

Table 3. Accumulated methane production (mL CH_4/ g DM [1]) and methane production per DMD [3] of different macroalgae with potential use in ruminant diets.

Item	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [2]	*p*-Value
3 h	0.33 [b]	0.03 [b]	0.25 [b]	0.27 [b]	5.26 [a]	0.408	0.0001
6 h	0.33 [b]	0.03 [b]	0.25 [b]	0.27 [b]	12.65 [a]	1.092	0.0001
9 h	1.99 [b]	0.09 [b]	0.85 [b]	3.88 [b]	20.46 [a]	2.847	0.0024
12 h	3.12 [b]	0.15 [b]	0.85 [b]	4.49 [b]	27.23 [a]	3.357	0.0009
24 h	4.53 [c]	0.18 [d]	0.85 [d]	13.02 [b]	64.41 [a]	0.588	0.0001
ml CH_4/g DMD [3]	6.61 [c]	0.26 [c]	2.04 [c]	90.18 [b]	144.32 [a]	4.787	0.0001

[1] mL CH_4/g incubated DM. [2] SEM: Standard error of pooled means. [3] mL CH_4/ g DMD 24 h. [a–d] Means within a row with different superscripts differ ($p \leq 0.05$).

The water retention expressed both in terms of time (g Water/1 g DM sample) (Table 4) and percentages (Table 5) was higher in macroalgae than in alfalfa hay. However, the highest water retention was for *G. skottsbergi*, followed by *M. pyriphera* ($p \leq 0.05$).

Table 4. Water retention (g Water/1 g DM sample) with respect to the initial weight of different macroalgae with potential use in ruminant diets.

Time (h)	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [1]	*p*-Value
0 h	11.34 [a]	9.40 [ab]	9.12 [ab]	7.15 [bc]	6.26 [c]	0.554	0.0006
3 h	10.47 [a]	8.09 [ab]	8.01 [ab]	6.18 [bc]	4.994 [c]	0.479	0.0001
6 h	9.86 [a]	7.48 [b]	7.08 [b]	5.30 [bc]	3.90 [c]	0.386	0.0001
9 h	9.49 [a]	6.99 [b]	5.70 [bc]	4.42 [c]	2.77 [d]	0.338	0.0001
12 h	9.14 [a]	6.63 [b]	4.51 [c]	3.70 [c]	1.80 [d]	0.284	0.0001
24 h	8.15 [a]	5.50 [b]	2.44 [c]	1.77 [c]	0.77 [c]	0.456	0.0001
36 h	7.40 [a]	4.63 [b]	2.52 [c]	1.80 [c]	0.32 [d]	0.193	0.0001
48 h	6.61 [a]	3.79 [b]	1.75 [c]	1.19 [c]	0.02 [d]	0.183	0.0001

[1] SEM: Standard error of pooled means. [a–d] Means within a row with different superscripts differ ($p \leq 0.05$).

Table 5. Water retention (%) of different macro algae with potential use in ruminant diets.

Time (h)	*G. skottsbergi*	*M. pyriphera*	*L. flavicons*	*U. lactuca*	Alfalfa Hay	SEM [1]	*p*-Value
0 h	100	100	100	100	100	0.000	0.9899
3 h	92.50 [a]	86.20 [b]	87.83 [b]	86.40 [b]	79.80 [c]	0.889	0.0001
6 h	87.53 [a]	79.70 [ab]	77.60 [b]	74.13 [b]	62.30 [c]	1.983	0.0001
9 h	84.36 [a]	74.70 [a]	62.56 [b]	61.86 [b]	44.30 [c]	2.147	0.0001
12 h	81.30 [a]	70.86 [b]	49.50 [c]	51.70 [c]	28.93 [d]	1.995	0.0001
24 h	72.67 [a]	58.93 [a]	20.10 [b]	34.13 [b]	12.30 [b]	4.960	0.0001
36 h	65.96 [a]	49.80 [b]	27.73 [c]	25.20 [c]	5.10 [d]	1.637	0.0001
48 h	58.90 [a]	40.70 [b]	19.30 [c]	16.63 [c]	0.40 [d]	1.366	0.0001

[1] SEM: Standard error of pooled means. [a–d] Means within a row with different superscripts differ ($p \leq 0.05$).

3. Discussion

Sustainably meeting the global quest for ruminant intensification while tackling issues including global warming, land degradation, and food–feed–fuel competition dictates the urgent need for alternative feed resources [1,2]. Recently, it has been well established that promoting macroalgae as a dietary ingredient for ruminants could sustain the production systems while mitigating methane emissions and adapting to climate change [11,12,17].

The present results on the chemical composition of macroalgae were in line with the previous reports [18–20]. The nutritional composition of different marine algae has been frequently studied [17,20]. In line with our results, a recent meta-analysis [8] of 47 published papers (25 in vitro and 22 in vivo studies) with a wide range of macroalgae including 46 species of brown, green, and red macroalgae revealed the average content of organic matter (OM), CP, NDF, and ADF to be 734.2, 189.2, 321.3, and 208.5 g/kg DM, respectively. Min et al. [17] also reported the CP (7.8 to 38.1% DM), NDF (16.6 to 43.1% DM), ADF (6.6 to 13.1% DM), and EE (0.3 to 3.9% DM) levels in eight macroalgae species of all brown, green, and red classes, which confirmed our data. It should also be acknowledged that the chemical composition bioactive content of macroalgae is influenced by their taxonomic classification (brown, green, or red), and varies among different genera and species. Seasonal variations may also affect these compositions during the growth or harvesting periods [2,20]. All four algae species examined in our research demonstrate acceptable chemical compositions, especially as a protein source; however, they should be incorporated into a total mixed ration (TMR) to elucidate their potential benefits.

Our data on the cumulative gas production of four macroalgae species after 48 h (20.97–58.68 mL/g DM) were in line with previous studies, where four tropical macroalgae (namely, *Laminaria* sp., *Padina australis*, *Gracilaria* sp., and *Eucheuma cottonii*) produced total gas values of 28.50–36.63 mL/g DM in a 48 h incubation period [21]. The total 72 h gas yield of three marine algae, *Macrocystis pyrifera*, *Ulva* spp., and *Mazzaella* spp., have been reported to be 63.4, 44.37, and 30.33, respectively [20]. Confirming out results, it has been demonstrated that marine algae have relatively low ruminal degradability and gas production, primarily due to their high ash content, which reduces the organic matter [18]. In the present study, *U. lactuca* was superior to other tested macroalgae in terms of total and potential gas production. This might be due to its higher soluble components (OM at 64.19% DM and CP at 18.59% DM) and the low ADF (9.60% DM), as described by Hidayah et al. [20]. The high NDF and ADF content may, in other tested macroalgae, contribute to the reduced gas production [2]. Min et al. [17] reported that the in vitro DM digestibility of macroalgae species after 96 h of incubation varied between 27.9% and 94.6% DM, which agrees with the current findings. A meta-analysis [8] of 25 in vitro papers of all brown, green, and red macroalgae classes revealed that DM digestibility ranges from 44.98% to 47.9%. However, in the present study, the DMD96 of *U. lactuca* was lower (14.72% DM) than that of the other tested macroalgae. In confirmation, Zitouni et al. [22] reported that the *U. lactuca* is characterized by high contents of minerals and CP, which contribute enormously to biomass production rather than gas production. It has been also well established that some algae contain polysaccharides, carrageenans, alginate, fucoidans, agar, ulvans, xylans, laminarin,

and florideans starch, which limits the availability of nutrients for rumen microbiota [23]. In our study, the potential of gas production (parameter b) and gas production rate (parameter c) were in line with those reported by Hidayah et al. [21] for different brown and red marine algae. Notably, Lee-Rangel et al. [20] evaluated three different algae species and showed that the in vitro gas production and DMD were higher for *M. pyrifera* and *Ulva* spp. compared with those for *Mazzaella* Spp. Also, CH_4 (%) at 48 h of mitigation with *M. pyrifera* (47.7%) < *Ulva* spp. (61.0%) < *Mazzaella* spp. (71%) indicated the superiority of *M. pyrifera* in ruminant feeding.

The CH_4 mitigatory impacts of marine algae have been frequently tested both in vivo and in vitro [8]. An in vivo study found that the supplementation of 0.5% and 1% of *Asparagopsis armata*, a red macroalga, to Holstein cows decreased CH_4 production by 26% and 67%, respectively [24]. Similarly, an in vitro experiment reported that the addition of *A. taxiformis* and *Z. farlowii* (at 5% DM of diet) reduced the microbial methane production by up to 78% and 11% after 48 h of incubation, respectively [25]. The addition of *Asparagopsis taxiformis* and *Asparagopsis armata* to a grass-basal substrate linearly decreased methane production throughout the 72 h in vitro fermentation [26]. Likewise, the in vitro anti-methanogenic effect of *Asparagopsis taxiformis* has been reported to be 84.7 and 99% at inclusion levels of 1 and 2% on an OM basis, respectively [27]. Dietary inclusions of *Bonnemaisonia hamifera* (a red seaweed) at 2.5%, 5.0%, and 7.5% of grass silage OM also reduced the in vitro CH_4 production (13.5, 14.5, and 8.8%, respectively) and gas production (mL/g OM) (12.5, 11.7 and 13.7%) compared with the control [19]. The results of another in vitro batch culture suggest that supplementation with red seaweed extracts altered the microbiota, leading to the acceleration of propionate production and a reduction in CH_4 production [28]. It has been well established that marine algae can be rich in halogenated aliphatic organic compounds—in particular, Phlorotannin and Organobromines including bromomethane (methyl bromide; CH_3Br) and bromoform ($CHBr_3$), which are recognized for their ability to inhibit microbial methanogenesis, thereby affecting the production of methane in rumen fermentation processes [25,27]. The anti-methanogenic action of bromoform is attributed to its interference with the cobamide-dependent methylation process, which is crucial for the formation of coenzyme-M, a key component in the final stage of methane production [27]. In addition, phlorotannin is recognized for modifying the population of cellulolytic bacteria, methanogenic archaea, and methanogens associated with ciliate protozoa [29]. It has been well documented [14] that phlorotannins reduce CH_4 emissions through direct interactions with methanogenesis, but further studies are necessary to elucidate the mechanisms of action. Other bioactive substances in macroalgae such as peptides, carbohydrates, lipids, saponins, sulfonated glycans, and bacteriocins can also contribute to mitigation methane emissions [9]. Furthermore, algae can possess high levels of ether extracts and may contain elevated levels of long-chain polyunsaturated fatty—in particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These are associated with decreased CH_4 production [20]. Overall, the evidence indicates that the secondary metabolites present in macroalgae serve to mitigate methane production during the enteric fermentation in the rumen [2,9,13]. However, it should be noted that these are accompanied by several limitations, including the limited studies and unclear mechanisms regarding how different macroalgae species impact rumen fermentation, which is a crucial factor to consider when examining CH_4 emissions and production performance in ruminants [2,8]. In summary, the current in vitro findings suggest that *M. pyriphera* and *L. flavicons* had not only acceptable CP contents and DMD but could also serve as effective feed additives for reducing methane in ruminants due to their eco-friendly local sourcing with a low carbon footprint. On the contrary, *U. lactuca* shows lower methane mitigation impacts and had lower DMD, and *G. skottsbergi* presents a low CP content, high water retention, and low rumen fermentation compared with the rest of the tested algae, which could disfavor animal performance.

4. Materials and Methods

All experimental methods and procedures were reviewed and approved by the Professional Committee for the Standardization of Experimental Animals of the Universidad de Magallanes (Chile) and the Universidad Autonoma del Estado de Mexico (project ID: 6663/2022 SF).

Four different macroalgae of all classes were used, including brown (*Lessonia flavicans* and *Macrocystis pyrifera*), red (*Gigartina skottbergii*), and green (*Ulva lactuca*). The study area is located in Laredo Bay (52°57′ S–70°51′ W), belonging to the Universidad de Magallanes, located on the west coast of the eastern sector of the Strait of Magellan, 25 km north of the city of Punta Arenas. The average surface water temperature ranges from 5.8 °C in winter to 10.5 °C in summer, while the average surface salinity fluctuates between 34.3 psu in summer and 36.0 psu in winter. The algae were collected throughout the bay for three weeks during the month of May 2023 and then dehydrated at 60 °C, 48 h, for this purpose. A total of 1 kg of the dry matter of each seaweed was sieved with the Standard Test Sieve of 4 mm N°5 (brand W.S. Tyler, USA, STATE). Subsequently, it was chopped in pieces of 1 mm in diameter for its later use.

Rumen fluid (300 g liquid and 200 g solid phases, approximately) was collected 2 h after morning feeding from two ruminal cannulated non-lactating beef cows (5 years old, 577 kg average body weight) and were mixed from one sample, filtered through four layers of cheesecloth, and immediately transported to the laboratory in pre-warmed thermo flasks. The donors were fed a maintenance diet (consisting of 60% corn silage and 40% concentrate, 16% CP, 2.8 Mcal ME/kg DM) with free access to water. The in vitro batch culture was conducted according to the procedure described by Theodorou et al. [30]. Briefly, 0.8 g of each of the four macroalgae and alfalfa hay (control) was added to 125 mL flasks (three flasks per treatment), with 100 mL of the inoculums consisting of ruminal fluid and buffer solution with three incubation batches (i.e., a total of nine replicates per diet). All flasks were randomly placed in a water bath and a continuous water bath at 39 °C, the gas volume (ml gas/g DM) was recorded at 3, 6, 9, 12, 24, 36, 48, 72, and 96 h using a pressure transducer (model 8804 HD), and a set of appropriate blanks and standards were included. To measure gas production kinetics, the data (mL/g DM) were fitted according to Krishnamoorthy et al. [31], using the following model: $GP = b\ (1 - e^{-ct})$, where GP = Gas production (mL gas/g DM); b = total gas production (mL gas/g DM); c = degradation rate compared with the time (hours); and t = time (h).

After the incubation period (96 h), dry matter degradability (DMD96 mg/100 mg) and relative gas production (RGP, ml gas 96 h)/(mg/100 mg DMD96) were measured. The concentrations of short chain fatty acids (SCFA, mmol/200 mg) and microbial crude protein (MCP, mg/g) were determined according to Blümmel et al. [32]. The ammonia nitrogen ($N\text{-}NH_3$) concentration was measured using the phenol hypochlorite method [33].

For CH_4 determination, glass syringes were filled under anaerobic conditions with 100 mL of a 1:9 mixture of rumen inoculum and incubation solution in a total of nine replicates per treatment, and three incubation runs were performed. CH_4 production was determined at 6, 12, and 24 h for this purpose, and a 1 mL aliquot of gas was obtained using a three-stage stopcock [34]. This sample was then diluted at a ratio of 1:100, and each sample of gas was methodically passed through a CH_4 detection device (PANGEA brand; Model PHG100, manufactured in China). The resulting three readings from each syringe were recorded in parts per million (ppm) and expressed in ml CH_4 accumulated/g DM incubated.

Water retention was also assessed according to Wang et al. [35]. Then, 1 g DM samples were weighed on Whatman paper filters, excess in funnels was collected, 25 mL of water was added, weight differences were recorded at intervals (0, 1, 2, 3, 6, 9, 12, 24, 36, and 48 h) at 20 °C, and a total of nine replicates per treatment in three runs were performed.

All samples were analyzed for DM, ether extract (EE), ash, and crude protein (CP) contents following AOAC [36] standards. Neutral detergent fiber (NDF), acid detergent

fiber (ADF) and acid detergent lignin (ADL), adjusted for ash content, were determined according to Van Soest et al. [37].

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) in a completely randomized design (CRD). The data were screened for normality using the UNIVARIATE procedure and were then analyzed using the MIXED procedure with treatments as a fixed factor and experimental runs as a random factor. The results were presented as least square means (LSM) with pooled standard errors. Differences in the means among the experimental groups were estimated using Tukey's test. Significance was set at $p \leq 0.05$, while tendencies were detected at $0.05 < p < 0.10$.

5. Conclusions

Here we investigated the potential of four species of sub-Antarctic macroalgae for their chemical composition, in vitro gas production, and CH_4 mitigation. The highest and lowest crude protein contents were for *U. lactuca*, and *G. skottsbergi*, respectively. *G. skottsbergi* and *M. pyriphera* showed the highest dry matter degradability at 96 h and microbial crude protein. All four tested algae produced lower amounts of methane compared to alfalfa hay. After 24 h of incubation, *M. pyriphera*, *L. flavicons*, *G. skottsbergi*, and *U. lactuca* reduced CH_4 by 99.7%, 98.6%, 92.9%, and 79.8%, respectively, when compared to the control. The current results suggest that *M. pyriphera* and *L. flavicons* are promising feed additives for ruminants with eco-friendly production and acceptable CP content and DMD that could effectively mitigate CH_4 emissions. However, future studies are suggested to evaluate the effect of these macroalgae in vivo to ensure that farmers have a sufficient incentive to implement such strategies.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Individuals interested in accessing the data may request them from the authors by providing a justification, and the authors will provide them at their convenience.

Conflicts of Interest: The authors declare no conflicts of interest.

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