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Effects of *Gelidium amansii* extracts on *in vitro* ruminal fermentation characteristics, methanogenesis, and microbial populations

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Objective: *Gelidium amansii* (Lamouroux) is a red alga belonging to the family *Gelidaceae* and is commonly found in the shallow coasts of many East Asian countries, including Korea, China, and Japan. *G. amansii* has traditionally been utilized as an edible alga, and has various biological activities. The objective of this study was to determine whether dietary supplementation of *G. amansii* could be useful for improving ruminal fermentation.

Methods: As assessed by *in vitro* fermentation parameters such as pH, total gas, volatile fatty acid (VFA) production, gas profile (methane, carbon dioxide, hydrogen, and ammonia), and microbial growth rate was compared to a basal diet with timothy hay. Cannulated Holstein cows were used as rumen fluid donors and 15 mL rumen fluid: buffer (1:2) was incubated for up to 72 h with four treatments with three replicates. The treatments were: control (timothy only), basal diet with 1% *G. amansii* extract, basal diet with 3% *G. amansii* extract, and basal diet with 5% *G. amansii* extract.

Results: Overall, the results of our study indicate that *G. amansii* supplementation is potentially useful for improving ruminant growth performance, via increased total gas and VFA production, but does come with some undesirable effects, such as increasing pH, ammonia concentration, and methane production. In particular, real-time polymerase chain reaction indicated that the methanogenic archaea and *Fibrobacter succinogenes* populations were significantly reduced, while the *Ruminococcus flavefaciens* populations were significantly increased at 24 h, when supplemented with *G. amansii* extracts as compared with controls.

Conclusion: More research is required to elucidate what *G. amansii* supplementation can do to improve growth performance, and its effect on methane production in ruminants.

Keywords: *Gelidium amansii*; Growth Rate; *In vitro* Fermentation; Microbial Growth

INTRODUCTION

Algae are an economically important and underexploited plant resource, providing potential biomass for animal feed and human foods in recent years. In fact, algae have a broad range of primary metabolites, including polysaccharides, polyphenols, amino acids and minerals [1,2] which can help improve growth rate and feed conversion efficiency [3,4]. Animal nutritionists have been studying manipulation of the rumen microbial ecosystem to reduce methane emission without adverse effects on rumen function. Methane emissions from greenhouse gases are low, but one molecule of methane affects 21 times more than one molecule of carbon dioxide. There is a need to identify feed additives to modify ruminal fermentation characteristics and increase the efficiency of feed utilization, whilst inhibiting ruminal methanogenesis.

Gelidium amansii (*G. amansii*) is a red alga belonging to the family *Gelidaceae* and is found in shallow coastal areas of many East Asian countries including North and South Korea, China, and Japan. Moreover, *G. amansii* has various biological activities, including antioxidant, anti-

tumor, cytotoxicity and immunomodulation effects [5,6].

Many studies have reported the composition and properties of algae, describing novel functional food ingredients, which may improve animal metabolic function and feed quality [3]. However, few papers have determined the potential of *G. amansii* effects on ruminal fermentation characteristics by *in vitro* gas production techniques. Such techniques have been applied to study fermentation kinetics relative to feed composition, and allow for rapid screening of a large number of feed additives that may have effects on fermentation process [7]. Previous studies have shown that algae similar to *G. amansii* are effective in methane reduction [8,9]. Therefore, the objective of this study was to determine the potential effects of dietary supplementation of *G. amansii* on *in vitro* fermentation parameters including total gas production, volatile fatty acid (VFA) composition, gas profile (methane, carbon dioxide, hydrogen, and ammonia) and microbial growth rate, according to changes occurring with microbial diversity, as compared to basal diet with timothy fermentation. The results could help determine whether dietary supplementation of *G. amansii* could be useful for improving ruminal fermentation.

MATERIALS AND METHODS

Preparation of *G. amansii* extract

Air-dried *G. amansii* samples were obtained from the Jeju Biodiversity Research Institute (JBRI, Jeju, Korea), and were stored under dry and dark conditions. Washed samples were cut into small pieces, freeze dried and crushed. Crushed powder was extracted with 80% EtOH at room temperature, using an ultrasonic cleaner (Branson Ultrasonics corporation, Danbury, CT, USA) and the EtOH eluate solution was filtered through Whatman No. 1 filter paper and concentrated under vacuum.

In vitro fermentation design

One cannulated Holstein cows (450±30 kg) was used as rumen fluid donors and provided with *ad libitum* access to water and a mineral-vitamin block. Timothy and commercial concentrate ratio (60:40, w/w) were fed twice daily at 09:00 and 17:00, equivalent to 2% of body weight. The rumen fluid collected before morning feeding was strained through four layers of cheese gauze, and the rumen contents were diluted by addition of artificial saliva and maintained at 39°C. All experimental protocols used in this research were approved and maintained in accordance with the Guide for the Animal Care and Use Committee of Gyeongsang National University (Jinju, Korea).

Dietary treatments were as follows: control (CON), basal diet (without *G. amansii* extract); treatment 1 (TRT 1), CON+1% *G. amansii* extract; treatment 2 (TRT 2), CON+3% *G. amansii* extract; and treatment 3 (TRT 3), CON+5% *G. amansii* extract as basis of substrate (timothy). The chemical composition of timothy was measured to contain the following: amount of moisture, 8.87%; crude protein, 13.37% ether extracts, 2.25%; crude fiber, 21.87%; crude ash, 8.62%; neutral detergent fiber, 53.18%; and acid detergent fiber, 30.57% as percentage of dry matter (DM).

Fifteen mL of rumen fluid: artificial saliva mixture (McDougall, 1:2, v/v) was dispensed into 50 mL serum bottles, containing 300 mg of timothy for CON and *G. amansii* extract for TRTs (3 mg for TRT 1, 9 mg for TRT 2 and 15 mg for TRT 3). The serum bottles were sealed under anaerobic conditions and capped with a butyl rubber stopper with an aluminum cap and then incubated in a gently shaking incubator (Jeio Tech, SI-900R, Daejeon, Korea; 120×rpm) at 39°C for 72 h. The *in vitro* fermentation experiment was a completely randomized block design and performed in triplicate, using 60 serum bottles (4 treatments×5 incubation times×3 replicates).

Analysis of gas profiles and ruminal fermentation characteristics

Total gas production was measured according to the method by Theodorous who suggested that the head space gas pressure should be measured using a detachable pressure transducer and a digital readout voltmeter (Laurel Electronics, Inc., Costa Mesa, CA, USA) after removing serum bottles from a shaking incubator. For gas profiling during 72 h, the transducer was connected to the inlet of a disposable Luer-lock three-way stopcock and then gas pressure in the headspace above the culture medium was read from the LED display unit after insertion of a hypodermic syringe needle. Furthermore, another outlet was connected to a gas chromatograph (HP 5890, Agilent Technologies, Santa Clara, CA, USA) using a TCD detector with a Carboxen-1006 Plot capillary column (30 mm×0.53 mm, Supelco, Bellefonte, PA, USA) for detection of methane, carbon dioxide and hydrogen.

Following gas profile analysis, serum bottles were uncapped and the culture medium was subsampled for pH analysis (MP230, Mettler-Toledo, Columbus, OH, USA), ammonia and VFA concentration. Ammonia concentration was measured as optical density (OD) values using a UV/VIS spectrophotometer (Model 680, Bio-Rad laboratories, Hercules, CA, USA) at 630 nm after sample preparation. For VFA measurement, culture medium was centrifuged at 3,000 rpm for 3 min and then supernatants were filtrated using a 0.2 µM disposable syringe filter (Whatman Inc., Clifton, NJ, USA). VFA analysis was performed with high performance liquid chromatography (HPLC, Agilent-1200, Waldbronn, Germany) using a UV/VIS detector with a MetaCarb 87H column (300 mm×7.8 mm, Varian, Palo Alto, CA, USA).

In vitro DM disappearance rate was determined using the nylon bag digestion method. After incubation, the nylon bag containing the substrate was washed twice in a water-bath equipped with a Heidolphs Rotamax 120 (Heidolph Instrument, Nuremberg, Germany) at 100 rpm for 30 min and then oven dried at 60°C to a constant weight. DM disappearance was defined as weight loss before and after incubation in the serum bottle.

Microbial growth performance

At the end of each fermentation period, samples were centrifuged at 3,000 rpm for 3 min to remove feed particles and the supernatants were re-centrifuged at 14,000 rpm for 3 min to pellet. Supernatants were divided for protein and glucose analysis. The protein was evaluated as OD value at 595 nm measured by a spectrophotometer (Model 680, Bio-Rad Laboratories, USA), using the dye binding properties of Coomassie Blue G-250. For glucose measurement, 200 μL of supernatant and 600 μL of 3,5-dinitrosalicylic acid solution were mixed and incubated for 5 min in a boiling water bath. The glucose concentration was determined as OD value at 595 nm using a microplate reader (Model 680, Bio-Rad Laboratories, USA). After discarding the supernatants, pellets were washed with sodium phosphate buffer (pH 6.5) and repeated three more times. The growth rate of total microorganisms was evaluated as OD value at 550 nm as measured by spectrophotometer (Model 680, Bio-Rad Laboratories, USA).

Quantitative real-time polymerase chain reaction

Samples were placed in screw-capped tubes containing silica beads for DNA extraction with a high-speed reciprocal shaker, following a modified bead-beating protocol with a Soil kit (Macherey-nagel, Düren, Germany). Briefly, a 1.0-mL aliquot of the incubated culture solution was centrifuged at 3,000×rpm, and then placed in a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) to determine nucleic acid concentrations.

Previous reports provided primers and thermocycling protocols used for amplification of general bacteria, ciliate protozoa, methanogenic archaea, *Fibrobacter succinogenes* (*F. succinogenes*), *Ruminococcus albus* (*R. albus*), and *Ruminococcus flavefaciens* (*R. flavefaciens*) were from previously published reports [10-13].

Quantitative real-time polymerase chain reaction (PCR) assays (CFX96 Real-Time system; Bio-rad, USA) were performed with the SYBR Green Supermix (QPK-201, Toyobo Co., LTD., Tokyo, Japan), following Denman and McSweeney [14] and Denman et al [11]. Abundance of these microbes was determined by the following equation:

Relative change in gene expression
$$= 2 - \Delta Ct(Target) - \Delta Ct(Control)$$

Where Ct represents threshold cycle. All quantitative (q) PCR reaction mixtures (final volume of 20 μL) contained forward and reverse primers, the SYBR Green Supermix and DNA template. Table 1 shown the PCR amplification of the target DNA, included the annealing and the extension temperature, was conducted following the same conditions described in the PCR primer references [10-13].

Statistical analysis

All experimental data were analyzed using the general linear model procedure of SAS [15] as a completely randomized block design. The effects of supplementation of *G. amansii* extract on pH, total gas production, DM disappearance, gas profiles, VFA profiles and microbial growth were compared to CON and significant differences were conducted using polynomial regression to measure the linear and quadratic effects of increasing concentrations of *G. amansii* extract. Variability in the data was expressed as standard error of the mean and p<0.05 was considered to be statistically significant, whereas p<0.10 was considered a tendency.

RESULTS

In vitro fermentation characteristics (cumulative pH, gas production, dry matter disappearance rate)

The effect of dose response of *G. amansii* extract on *in vitro* fermentation parameters is shown in Table 2. *G. amansii* extracts increased pH correlating positively with *G. amansii* extract concentration. With regards to cumulative gas production by mixed ruminal microorganisms, *G. amansii* extracts reduced cumulative gas production at 3 h, but increased it at 12, 24 and 72 h as compared with CON, respectively. Lastly, DM disappearance with supplementation of *G. amansii* extracts was lower than that of CON during the whole experimental period.

Volatile fatty acid profile and acetic acid/propionic acid ratio

The effect of dose response of *G. amansii* extract on VFA profiles of acetate, propionate, butyrate, and acetic acid/propionic acid (A/P) ratio are shown in Table 3. The supplementation of *G. amansii* extract increased total VFA concentration, acetate

Table 1. Polymerase chain reaction primer sets for real-time polymerase chain reaction assays

Target species	Primer sequences (5′→3′)	References
Ciliate-associated methanogens	F: GAG CTA ATA CAT GCT AAG GC	Skillman et al [12]
	R: CCC TCA CTA CAA TCG AGA TTT AAG G	
Methanogenic archaea	F: TTC GGT GGA TCD CAR AGR GC	Denman et al [11]
	R: GBA RGT CGW AWC CGT AGA ATC C	
Fibrobacter succinogenes	F: GTT CGG AAT TAC TGG GCG TAA A	Denman and McSweeney [10]
	R: CGC CTG CCC CTG AAC TAT C	
Ruminococcus albus	F: CCC TAA AAG CAG TCT TAG TTC G	Koike and Kobayashi [13]
	R: CCT CCT TGC GGT TAG AAC A	
Ruminocuccus flavefaciens	F: CGA ACG GAG ATA ATT TGA GTT TAC TTA GG	Denman and McSweeney [10]
	R: CGG TCT CTG TAT GTT ATG AGG TAT TAC C	

Table 2. Effect of *Gelidium amansii* extract on *in vitro* cumulative pH, gas emission, dry matter disappearance by mixed rumen microbial fermentation

Incubation (h)	Treatments[1]				SEM	p-value	
	CON	TRT 1	TRT 2	TRT 3		Linear	Quadratic
pH							
3	7.27	7.34	7.34	7.35	0.02	0.0693	0.2109
12	7.18	7.07	7.10	7.15	0.05	0.8320	0.1610
24	6.83	6.83	6.95	6.97	0.03	0.0078	0.6507
48	6.63	6.52	6.72	6.80	0.05	0.0084	0.0762
72	6.39	6.37	6.50	6.63	0.04	0.0012	0.0693
Gas emission (mL/g DM)							
3	192.86	185.36	182.14	184.73	3.17	0.0871	0.1501
12	197.30	212.03	210.65	208.59	2.08	0.0082	0.0038
24	223.43	239.38	226.76	242.34	8.39	0.2739	0.9830
48	257.54	288.01	273.91	273.28	5.12	0.1864	0.0162
72	278.61	291.49	290.23	294.24	2.53	0.0037	0.1172
DM disappearance (%)							
3	25.81	26.66	24.66	24.39	0.30	0.0034	0.1239
12	29.18	28.44	26.04	24.62	0.66	0.0003	0.4852
24	38.48	32.81	27.87	25.81	0.65	<0.0001	0.0099
48	40.85	42.19	35.52	31.84	0.49	<0.0001	0.0009
72	46.05	45.06	39.76	35.79	0.78	<0.0001	0.0938

SEM, standard error of the mean; DM, dry matter.
[1] Dietary treatments were as follows: CON, basal diet (without *G. amansii* extract); TRT 1, CON+1% *g. amansii*; TRT 2, CON+3% *G. amansii*; TRT 3, CON+5% *G. amansii* as basis of substrate (timothy).

concentration, propionate concentration, butyrate concentration and A/P ratio as compared with CON, respectively.

Emission gas profile (methane, carbon dioxide, and ammonia)

The effect of dose response of *G. amansii* extract on the gas profiles of methane, carbon dioxide and ammonia are shown in Table 4. *G. amansii* extract reduced methane emissions relative to CON at 3 h, carbon dioxide emission was increased relative to CON at 12 h. Futhermore, *G. amansii* extract incerased hydrogen, ammonia emission as compared with CON.

In vitro ruminal change in microbial diversity

The effect of dose response of *G. amansii* extract on microbial growth rate, protein and glucose concentration is shown in Table 5. The supplementation of *G. amansii* extract increased microbial growth rate and glucose concentration, while reducing protein concentration as compared with CON, respectively.

Changes in ruminal microbial diversity supplemented with *G. amansii* extracts are shown in Figure 1a (after 12 h incubation) and Figure 1b (after 24 h incubation), respectively. Supplementation with *G. amansii* extracts lead to an increase in the ciliate-associated methanogen population at 12 h, while reducing the methanogenic archaea population at 24 h as compared with CON, respectively. With regards to major fibrolytic microorganisms, *G. amansii* extracts reduced *R. albus* at 12 h, 24 h and *F. succinogenes* at 12 h, while increasing *R. flavefaciens* at 24 h as compared with CON, respectively.

DISCUSSION

Overall, *G. amansii* treatments did not appear to be detrimental to ruminal fermentation as assessed by *in vitro* fermentation parameters. In fact, *G. amansii* supplementation has the potential to assist in ruminant feeding for improved gas production and fermentation performance. For example, supplementation with *G. amansii* treatments produced pH values remaining in the proper range of 6.37 to 7.35, which is a suitable pH range for cellulose digestion (6.0 to 6.8), protein synthesis (6.3 to 7.4), proteolytic activity (6.5 to 7.0) and VFA productivity (6.0 to 6.6) as suggested by McCullough [16], as well as for ruminal microbial activity, which is not negatively affected within a pH range of 5.8 to 7.2 [17]. In addition, % DM disappearance after *G. amansii* supplementation was significantly reduced for the whole experimental period; however, total gas production was significantly increased at 12 and 72 h incubation as compared to CON, which may indicate a potential improvement to feed utilization efficiency [18]. However, when dietary fiber was included in *G. amansii* treatments, an increase of total gas production was observed without any reduction of % DM disappearance, which is in agreement with other algae studies involving dietary fiber [19].

In recent years, extensive studies investigating the potential use of terrestrial plants for nutritional manipulation of enteric methane production have been conducted. Interestingly, one study focusing on algae supplementation reported reduced methane production [20]; however, the current study provides evidence that *G. amansii* supplementation can significantly increase *in*

Table 3. Effect of *Gelidium amansii* extract on total volatile fatty acids (VFA), acetic acid, propionic acid, butyric acid and acetic acid/propionic acid (A/P) ratio by mixed rumen microbial fermentation

Incubation (h)	Treatments[1]				SEM	p-value	
	CON	TRT 1	TRT 2	TRT 3		Linear	Quadratic
Total VFA concentration (mM/g)							
3	71.73	74.56	83.62	88.68	2.50	0.0007	0.6669
12	83.97	97.84	98.48	100.26	2.95	0.0056	0.0746
24	106.85	109.28	112.60	131.66	6.19	0.0229	0.2161
48	113.99	121.27	133.06	142.44	4.36	0.0011	0.8150
72	123.79	136.95	148.77	161.87	4.45	0.0002	0.9948
Acetic acid concentration (mM/g)							
3	49.07	51.06	59.74	64.64	2.29	0.0006	0.5429
12	57.76	69.57	72.67	74.33	2.59	0.0019	0.0859
24	77.48	76.22	79.42	94.10	5.78	0.0742	0.2050
48	81.03	84.10	93.16	100.00	4.13	0.0072	0.6593
72	88.20	94.03	102.06	110.23	4.33	0.0050	0.7937
Propionic acid concentration (mM/g)							
3	14.07	14.62	14.59	14.87	0.51	0.3257	0.7921
12	17.10	17.74	15.71	16.01	0.48	0.0385	0.7420
24	19.33	21.31	21.17	23.02	0.87	0.0229	0.9407
48	22.70	24.45	26.58	27.65	0.66	0.0004	0.6204
72	24.96	29.86	31.58	34.71	0.89	<0.0001	0.3488
Butyric acid concentration (mM/g)							
3	8.60	8.88	9.30	9.17	0.42	0.2873	0.6399
12	9.10	10.53	10.11	9.92	0.51	0.4057	0.1543
24	10.03	11.75	12.01	14.53	0.50	0.0003	0.4396
48	10.26	12.71	13.31	14.78	0.32	<0.0001	0.1595
72	10.64	13.06	15.13	16.92	0.56	<0.0001	0.5874
A/P ratio							
3	3.49	3.49	4.11	4.38	0.22	0.0098	0.5547
12	3.38	3.92	4.63	4.64	0.14	<0.0001	0.0895
24	3.99	3.59	3.76	4.10	0.26	0.6899	0.1916
48	3.56	3.44	3.51	3.63	0.18	0.7539	0.5327
72	3.55	3.17	3.24	3.18	0.23	0.3341	0.4878

SEM, standard error of the mean.
[1] Dietary treatments were as follows: CON, basal diet (without *G. amansii* extract); TRT 1, CON+1% *G. amansii*; TRT 2, CON+3% *G. amansii*; TRT 3, CON+5% *G. amansii* as basis of substrate (timothy).

vitro methane and carbon dioxide emission. In particular, increased methane production may have partially been due to an alteration in microbial diversity with an increase in the protozoan population (ciliate-associated methanogens) [21], and a major member of the fibrolytic microorganism population, *R. flavefaciens* [22], resulting from *G. amansii* treatments as compared with CON. However, methanogenic archaea, *R. albus*, and *F. succinogenes* (two other major members of the fibrolytic microorganism population) populations were significantly reduced. Ciliate-associated methanogens may generate up to 37% of methane produced in the rumen [23]. Therefore, although an increase in ciliate-associated methanogens may help to explain the increase in methane production, a reduction in methanogenic archaea would counter it. With regards to the fibrolytic microorganism population, *R. flavefaciens* normally produces succinic acid as a major fermentation product together with acetic and formic acids, H_2, and CO_2. Additionally, *R. albus* is a very promising

bacterium to produce H_2 from energy forage, with the potential of utilizing the cellulosic and hemicellulosic biomass [24]. In contrast, *F. succinogenes* is a non-H_2-producing species. The increase in the *R. flavefaciens* population might be the culprit behind the increase in methane and CO_2 production. A previous study by Chaucheyras-Durand et al [25] showed that methane production was clearly reduced when the dominant fibrolytic species was a non-H_2-producing species, such as *F. succinogenes*, without significantly impairing fiber degradation and fermentations in the rumen. This was not the case in our study. As such, H_2 is of critical concern to the microbial ecosystem in ruminants. H_2 produced during enteric fermentation is the precursor of methane emission from ruminants, and the regulation of H_2, rather than methane, is the key to controlling ruminant methane emission. In addition, 80% of total enteric methane production is generated from carbon dioxide and hydrogen as a substrate [26], which supports the positive correlation observed with methane

Table 4. Effect of *Gelidium amansii* extract on *in vitro* methane, carbon dioxide, hydrogen and ammonia emission by mixed rumen microbial fermentation

Incubation (h)	Treatments[1]				SEM	p-value	
	CON	TRT 1	TRT 2	TRT 3		Linear	Quadratic
Methane emission (mL/g DM)							
3	19.24	16.83	15.19	15.73	0.89	0.0158	0.1364
12	20.15	23.12	37.96	32.34	2.15	0.0007	0.0811
24	47.37	68.32	63.34	62.90	3.40	0.0255	0.0136
48	72.79	87.12	74.40	70.76	5.90	0.4959	0.1665
72	79.32	93.90	86.45	94.21	5.72	0.1834	0.5669
Carbon dioxide emission (mL/g DM)							
3	25.71	23.08	29.63	39.12	15.66	0.0056	0.0121
12	78.06	79.89	101.93	68.73	15.00	0.0089	0.0870
24	119.81	151.22	114.46	113.64	22.80	<0.0001	0.6923
48	137.75	166.10	153.13	126.46	19.07	<0.0001	0.4606
72	166.33	206.01	181.97	164.13	24.02	<0.0001	0.1778
Hydrogen emission (mL/g DM)							
3	5.82	5.98	5.76	5.79	0.21	0.7523	0.7626
12	6.79	6.47	9.09	7.20	0.54	0.1533	0.1866
24	8.89	12.63	9.82	11.60	0.91	0.2281	0.3118
48	12.59	14.47	12.33	12.93	0.90	0.7888	0.4996
72	14.75	14.80	13.80	13.73	0.93	0.3567	0.9506
Ammonia emission (mg/dL)							
3	2.57	6.22	6.37	6.00	0.62	0.4365	0.4623
12	4.52	8.76	12.23	10.80	1.45	0.1563	0.2049
24	4.74	10.34	21.10	25.64	1.29	0.6709	0.0447
48	7.55	16.29	38.27	41.16	3.77	0.9044	0.7609
72	8.17	24.00	41.41	51.31	2.00	0.0284	0.1061

SEM, standard error of the mean; DM, dry matter.
[1] Dietary treatments were as follows: CON, basal diet (without *G. amansii* extract); TRT 1, CON+1% *G. amansii*; TRT 2, CON+3% *G. amansii*; TRT 3, CON+5% *G. amansii* as basis of substrate (timothy).

Table 5. Effect of *Gelidium amansii* on rumen microbial growth rate, protein and glucose concentration

Incubation (h)	Treatments[1]				SEM	p-value	
	CON	TRT 1	TRT 2	TRT 3		Linear	Quadratic
Microbial growth rate (OD at 550 nm)							
3	0.34	0.32	0.37	0.40	0.03	0.1555	0.4844
12	0.30	0.36	0.42	0.51	0.04	0.0083	0.7393
24	0.35	0.28	0.33	0.37	0.03	0.5089	0.0997
48	0.43	0.50	0.44	0.63	0.03	0.0022	0.0623
72	0.33	0.41	0.49	0.48	0.03	0.0045	0.1539
Protein concentration (mM/g)							
3	0.13	0.08	0.08	0.08	0.00	<0.0001	<0.0001
12	0.14	0.09	0.11	0.09	0.00	<0.0001	0.0029
24	0.14	0.12	0.12	0.11	0.01	0.0022	0.3432
48	0.15	0.13	0.13	0.11	0.02	0.0906	0.7485
72	0.17	0.13	0.13	0.12	0.02	0.0880	0.3965
Glucose concentration (mL/mg)							
3	0.14	0.15	0.15	0.17	0.01	0.0119	0.4220
12	0.15	0.16	0.17	0.18	0.01	0.0224	0.8394
24	0.16	0.18	0.25	0.22	0.02	0.0072	0.1809
48	0.18	0.19	0.28	0.28	0.05	0.1354	0.9197
72	0.19	0.23	0.29	0.30	0.04	0.0912	0.7791

SEM, standard error of the mean; OD, optical density.
[1] Dietary treatments were as follows: CON, basal diet (without *G. amansii* extract); TRT 1, CON+1% *G. amansii*; TRT 2, CON+3% *G. amansii*; TRT 3, CON+5% *G. amansii* as basis of substrate (timothy).
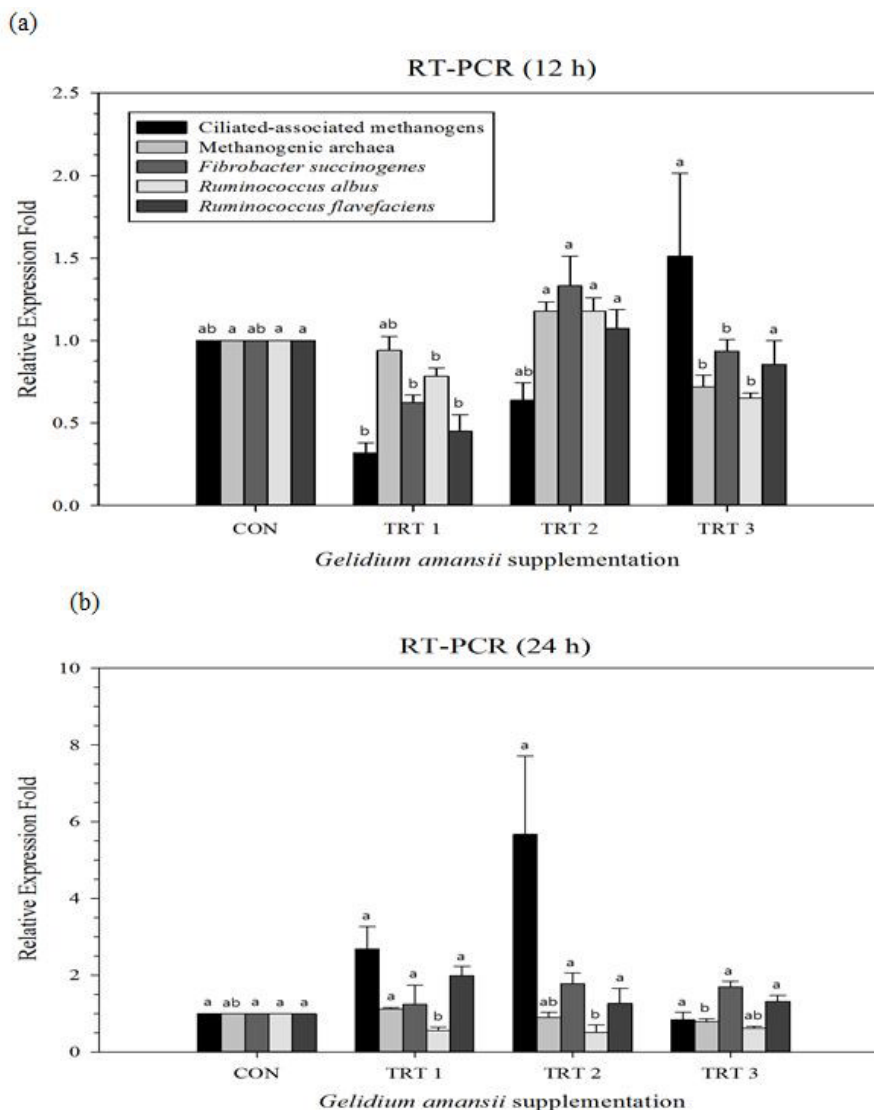
Figure 1. Relative quantification analysis of rumen microorganism populations under *in vitro* ruminal fermentation by the addition of marine alga *Gelidium amansii* after 12 h (a) and 24 h (b) incubation. Microorganism populations examined include ciliate-associated methanogens, methanogenic archaea, and cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*). Dietary treatments were as follows: CON, basal diet (without *G. amansii*); TRT 1, CON+1% *G. amansii*; TRT 2, CON+3% *G. amansii*; TRT 3, CON+5% *G. amansii* as basis of substrate (timothy). [ab] Means with different superscripts in the same row differ significantly (p<0.05).

and carbon dioxide production in our study. Increased methane emission is indicative of increased methanogen activity. The methanogens utilize mainly hydrogen and carbon dioxide, secondary fermentation products produced by rumen fermentation [27], as well as acetate, as a substrate for methanogenesis [28]. By removing hydrogen as the precursor of ruminal methane emission, methanogens allow the microorganisms involved in fermentation to function optimally and support the complete oxidation of substrates [29]. Overall, *G. amansii* supplementation resulted in increased methane production, which can be partially explained by increased methanogen activity (increased ciliate-associated methanogens) resulting from an increase in carbon dioxide production by the *R. flavefaciens* populations, acetate concentration and A/P ratio; however, all these parameters are still within opti-

mal fermentation conditions [30]. In addition to having an effect on methane and carbon dioxide, *G. amansii* supplementation also resulted in a significantly higher concentration of total VFA, acetate, propionate, butyrate, and A/P ratio being produced as compared to CON, demonstrating that fermentation was significantly affected. *G. amansii* supplementation resulted in a significantly increased amount of microbial growth at 12, 48 and 72 h, as compared to CON, which is in agreement with Ha et al [18], who suggested that rumen microorganisms need an adaptation period for changing environmental conditions of up to 6 h before their numbers increase, until nutrient depletion and waste products generated from microbial growth in the medium begin to inhibit their growth. Moreover, VFAs are released as the major end products of rumen microbial fermentation instead of glucose.

Propionate is the most abundant of the glucogenic acids and the predominant substrate for gluconeogenesis in ruminants [31]. Interestingly, both propionate and glucose concentration were significantly increased throughout the whole experimental period after *G. amansii* supplementation, demonstrating a positive correlation with one another. Overall, *G. amansii* supplementation resulted in a higher microbial growth rate, manifesting itself in the form of observed higher total gas and VFA production as compared with CON.

Finally, rumen ammonia concentration can vary depending on the proportion of feed protein and degradation rate. *G. amansii* supplementation resulted in a significantly higher ammonia concentration during the whole experimental period, which was still within the optimal ammonia concentration range for ruminal fermentation [32], thus demonstrating that rumen fermentation was not detrimentally affected by *G. amansii* supplementation. There was no correlation between ammonia amount and protein production observed in our study.

The objective of this study was to investigate and determine whether dietary supplementation of *G. amansii* could be useful for improving ruminal fermentation, as assessed by *in vitro* fermentation parameters. Overall, the results of our study indicate that *G. amansii* supplementation is potentially useful (i.e. may improve ruminant growth performance via increased total gas and VFA production), but does come with some undesirable effects. For example, *G. amansii* supplementation appears to increase methane production (increased methanogenic activity by ciliate-associated methanogens using increased H_2 and CO_2 being produced by an increased *R. flavefaciens* population), which is in disagreement with previous observations on Rhodophyta supplementation under *in vitro* fermentation conditions. More research is required to demonstrate and elucidate what *G. amansii* supplementation can do to improve growth performance and its effect on methane production in ruminants.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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