

Article

Effects of *Codium fragile* Extract Additive on In Vitro and In Vivo Methane Production and Fermentation Characteristics of Korean Native Steers (Hanwoo)

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Abstract

The present study was conducted to demonstrate the effects of *Codium fragile* extract on methane production using in vitro and in vivo experiments. An in vitro batch experiment was conducted to evaluate different inclusion levels of *Codium fragile* extract (0, 0.25, and 0.5% of substrate dry matter). Methane production significantly decreased in the 0.5% treatment ($p < 0.05$), whereas dry matter digestibility and total volatile fatty acid concentration were not significantly affected ($p > 0.05$). Based on the in vitro results, an in vivo feeding experiment was conducted using a 0.5% inclusion level of *Codium fragile* extract on Hanwoo steers. Methane emissions were significantly decreased by approximately 10% in steers fed *Codium fragile* extract ($p < 0.05$). In contrast, rumen fermentation characteristics, feed intake, average daily gain, and blood parameters were not significantly different between the treatments ($p > 0.05$). These results demonstrate that a dietary additive with 0.5% *Codium fragile* extract effectively reduced methane emissions without negatively affecting rumen fermentation and growth performance in Hanwoo steers.

Keywords: *Codium fragile*; methane reduction; rumen fermentation; Hanwoo

1. Introduction

Inhibiting enteric methane emissions from cows is a major challenge for sustainable livestock production. Methane contributes to greenhouse gas emissions and reduces intake during ruminal fermentation [1]. Methane is produced as a byproduct of anaerobic fermentation in the rumen, and its formation is linked to overall fermentation patterns and hydrogen availability [2]. Therefore, strategies that control rumen fermentation characteristics to reduce methane emissions without adversely affecting feed utilization are important.

Marine-derived feed additives have garnered increasing attention owing to their potential to influence rumen fermentation and inhibit methane production [3]. Seaweeds contain a wide range of bioactive compounds, including polysaccharides and phenolic compounds, which may affect fermentation characteristics and gas production [2]. Although certain seaweeds have demonstrated strong methane-suppressing effects, concerns regarding mineral accumulation, animal safety, and practical applicability have limited their widespread use [4,5]. Therefore, it is necessary to identify seaweed resources with methane mitigation potential.



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Codium fragile, a green macroalga commonly found in the coastal regions of East Asia, such as Republic of Korea and Japan, is traditionally consumed as food and is known to contain sulfated polysaccharides and antioxidant compounds [6]. Extracts produced from *Codium fragile* have exhibited antimicrobial and metabolic regulatory properties in several studies [7,8]. Recently, long-chain saturated and unsaturated fatty acids present in *Codium fragile* were shown to influence fermentation characteristics and gas production, suggesting their potential contribution to methane mitigation [9]. However, studies on the effects of *Codium fragile* extract on rumen fermentation characteristics and methane production are limited.

In vitro batch systems provide a controlled approach for evaluating the effects of feed additives on gas production and fermentation characteristics. This system is widely used for preliminary assessment of methane mitigation potential [10]. Nevertheless, in vitro findings alone are insufficient for predicting animal-level responses, as in vivo conditions involve complex digestive processes and physiological regulation [11]. Therefore, in vivo evaluation is essential to confirm the efficacy and safety of *Codium fragile* extract.

The objective of this study was to evaluate the effects of *Codium fragile* extract on methane production and rumen fermentation characteristics using in vitro and in vivo experiments. Specifically, the aims of this study were to (i) demonstrate the effects of different concentrations of *Codium fragile* extract on methane production and fermentation characteristics in an in vitro batch system and (ii) validate the effects of *Codium fragile* extract on methane emissions, rumen fermentation characteristics, and animal performance.

2. Materials and Methods

2.1. *Codium Fragile* Preparation and Extract

Fresh *Codium fragile* was obtained from a commercial farm located in Wando, Republic of Korea. The seaweed was dried in a forced-air drying oven at 60 °C for five days and subsequently ground using a grinder (HSB-HB100, Hwngso Agricultural Machinery Inc., Daegu, Republic of Korea) equipped with a 2-mm screen. The powdered material was immersed in 90% ethanol and extracted for 14 days. After extraction, the solvent was removed by freeze-drying for five days. The extraction yield was approximately 31% relative to the dry weight of the original material. The freeze-dried extract was then finely ground through a 1-mm screen and used for both the in vitro and in vivo experiments.

2.2. In Vitro Experiment

2.2.1. Animals and Diet Composition

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Republic of Korea (approval number: NIAS2023-0616). Rumen fluid was obtained from two rumen-cannulated Hanwoo steers (39 months old). The donor animals were fed 9.4 kg of concentrate and 2.4 kg of mixed hay daily (as-fed basis) at 10:00 and 15:00. Water and mineral blocks were available ad libitum. The ingredient composition and chemical characteristics of the diets are presented in Table 1. Feed samples were dried at 60 °C for 48 h and ground using a Cyclotec 1093 mill (FOSS, Suzhou, China) fitted with a 1-mm screen for the determination of dry matter content. The processed samples were stored at −20 °C until chemical analysis. Nutrient composition was determined according to AOAC procedures [12].

Table 1. Ingredients and chemical composition of in vitro experimental diets.

Item	Concentrate	Mixed Hay
Ingredients, % of dry matter		
Cracked corn	54.34	
Soybean hull	6.51	
Corn gluten feed	5.08	
Wheat bran	6.51	
Soybean meal	8.82	
Lupin	6.51	
Limestone	0.77	
Sodium bicarbonate	0.37	
Salt	0.37	
Vitamin and mineral mixture ¹	0.25	
Kentucky bluegrass		50.00
Tall fescue		50.00
Chemical composition, % of dry matter		
Crude protein	19.2	6.23
Ether extract	3.40	1.06
Neutral detergent fiber	24.3	64.1
Acid detergent fiber	10.8	40.5
Crude ash	6.90	7.59
Non-fiber carbohydrates ²	46.2	21.02

¹ Vitamin and mineral mixture: vitamin A, 2,650,000 IU; vitamin D3, 530,000 IU; vitamin E, 1050 IU; nicotinic acid, 10,000 mg; iron, 13,200 mg; manganese, 4400 mg; zinc, 4400 mg; copper, 2200 mg; iodine, 440 mg; cobalt, 440 mg.

² Non-fiber carbohydrates = 100 – (crude protein + ether extract + crude ash + neutral detergent fiber).

2.2.2. Incubation Conditions

Rumen fluid was collected from the donor steers approximately 1 h before the morning feeding. The collected fluid from both animals was combined, mixed thoroughly, and filtered through four layers of cheesecloth. The filtered rumen fluid was immediately transported to the laboratory for incubation.

The substrate consisted of 0.4 g concentrate and 0.1 g mixed hay (DM basis), corresponding to the diet fed to the donor animals. Rumen fluid was mixed with McDougall's buffer solution [13] containing 0.5 g L-cysteine at a ratio of 1:3 (v/v). The buffered rumen fluid was continuously flushed with CO₂ to maintain anaerobic conditions.

The substrate was placed into Ankom F57 filter bags (Ankom Technology Corp., Macedon, NY, USA). Treatments consisted of an additive with *Codium fragile* extract at 0, 0.25, or 0.5% of substrate dry matter. Each filter bag containing the substrate and extract was transferred to a 125-mL serum bottle containing 50 mL of buffered rumen fluid. The headspace of each bottle was flushed with CO₂ before sealing. Bottles were incubated in a shaking incubator at 39 °C and 120 rpm for 24 and 48 h. Each treatment was replicated four times, and the glass bottle served as the experimental unit. Bottles incubated for 24 and 48 h were prepared independently.

2.2.3. Sample Collection and Analysis

Following incubation (24 or 48 h), the gas produced in each bottle was collected into a 10-mL evacuated tube (BD Vacutainer, Eysins, Switzerland) using a syringe. Methane concentration in the collected gas samples was determined using a gas chromatograph (NL/450 GC, Bruker, Billerica, MA, USA) equipped with a GS-GASPRO capillary column (30 m × 0.320 mm; Agilent Technologies Inc., Santa Clara, CA, USA).

The pH of the fermentation fluid was measured using a pH meter (SevenEasy pH, Mettler-Toledo AG, Schwerzenbach, Switzerland). Residual rumen fluid was transferred to separate tubes for analysis of volatile fatty acids (VFAs), ammonia nitrogen (NH₃-N), and

rumen microbial populations. Samples intended for microbial analysis were immediately frozen in liquid nitrogen and stored until analysis.

After incubation, filter bags containing the substrates were dried at 60 °C for 48 h to determine in vitro dry matter digestibility (IVDMD), calculated based on the difference between the initial and residual dry matter.

The concentrations of VFAs and NH₃-N were determined following the procedures described by Erwin et al. [14] and Chaney and Marbach [15], with slight modifications. Briefly, 5 mL of centrifuged rumen fluid supernatant was mixed with 500 µL of 50% metaphosphoric acid (MPA) for VFA analysis or 500 µL of 25% MPA for NH₃-N determination and stored at −80 °C until analysis. For VFA analysis, samples were centrifuged at 14,000× g for 10 min at 4 °C, and the supernatant was analyzed by gas chromatography (6890N, Agilent Technologies, Wilmington, DE, USA) equipped with a Nukol fused-silica capillary column (15 m × 0.53 mm × 0.5 µm; Supelco Inc., Bellefonte, PA, USA). A calibration curve was prepared using a volatile-free acid standard mixture (CRM46975, Sigma-Aldrich, St. Louis, MO, USA).

For NH₃-N determination, samples were centrifuged at 14,000× g for 5 min at 4 °C. Subsequently, 20 µL of supernatant was mixed with 1 mL of phenol color reagent and 1 mL of alkali-hypochlorite reagent. The mixture was incubated at 37 °C for 15 min to allow color development. After adding 8 mL of distilled water, absorbance was measured at 630 nm using a UV spectrophotometer (Bio-Rad Benchmark Plus, Tokyo, Japan).

2.3. In Vivo Experiment

2.3.1. Experimental Design and Methane Gas Measurement

Eight Hanwoo steers (average body weight 333.1 ± 23.7 kg; 14 months old) were used in a 2 × 2 crossover design consisting of two treatments and two experimental periods. Each period lasted three weeks. The treatments were: a control diet without an additive (CON) and a diet added with 0.5% *Codium fragile* extract (CFE) on a dry matter basis. Diets were formulated with a concentrate-to-forage ratio of 7:3.

Animals were housed individually in pens (2.5 × 5.0 m) with free access to drinking water. Feed was offered at a level equivalent to 2% of body weight (DM basis) and provided twice daily at 10:00 and 15:00. The concentrate formulation was identical to that used in the in vitro experiment (Table 1). Each experimental period consisted of a two-week adaptation phase followed by a one-week measurement phase.

Methane emissions were monitored using the GreenFeed system (C-Lock Inc., Rapid City, SD, USA). Prior to data collection, CO₂ recovery tests were conducted for each period, yielding recovery rates of 99.14% and 99.17% for periods 1 and 2, respectively. Methane emissions were recorded for six consecutive days, with measurements obtained every three hours (16 measurements per animal). Feed intake was calculated as the difference between the amount offered and refusals on a dry matter basis. Average daily gain was determined from body weight measured at the beginning and end of each experimental period. A washout period of 14 days was applied between the two experimental periods.

2.3.2. Sample Collection and Analysis

At the end of each experimental period, rumen fluid and blood samples were collected four hours after the morning feeding. Blood samples (10 mL) were obtained from the jugular vein using EDTA-coated tubes (BD Vacutainer, Eysins, Switzerland). The samples were centrifuged at 2000× g for 15 min at 4 °C to obtain serum, which was then stored at −20 °C until analysis.

Serum biochemical parameters, including glucose, creatinine, blood urea nitrogen, inorganic phosphate, calcium, total protein, albumin, globulin, alanine aminotransferase,

alkaline phosphatase, gamma-glutamyl transferase, total bilirubin, cholesterol, amylase, and lipase, were analyzed using an IDEXX Catalyst DX analyzer (IDEXX Laboratories Inc., Cary, NC, USA).

Rumen fluid samples (approximately 200 mL) were obtained using a stomach tube system connected to a vacuum pump (Welch and Thomas, Mt. Prospect, IL, USA). The tube consisted of a rigid head (13 cm length, 3 cm diameter) attached to a flexible tube (120 cm length, 1 cm diameter). The collected rumen fluid was immediately used to measure pH, and samples were further processed for VFA and NH₃-N analysis using the same procedures described for the in vitro experiment.

2.4. Statistical Analysis

All data from the in vitro experiments were analyzed using the GLM procedure in SAS (Enterprise Guide 7.1; SAS Institute Inc., Cary, NC, USA). Incubation time and RP treatments were included in the statistical model, and fixed effects were considered. Differences among the treatment means were analyzed using Tukey's multiple-comparison test. All data were presented as least-squares means, and significance was declared at $p < 0.05$.

All data from the in vivo experiments were analyzed using the GLIMMIX procedure in SAS (Enterprise Guide 7.1, SAS Institute Inc., Cary, NC, USA). Treatment was considered a fixed effect, whereas period and cow were considered random effects. The statistical model used was as follows:

$$Y_{ijk} = \mu + T_i + P_j + C_k + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the fixed effect of treatment, P_j is the random effect of period, C_k is the random effect of steer, and ε_{ijk} is the residual error. To minimize potential carryover effects between treatments, a 14-day washout period was implemented between experimental periods. Differences among treatment means were determined using Tukey's multiple-comparison test. Data are presented as least-squares means. Significance and tendencies were declared at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

3. Results

3.1. In Vitro Experiment

The effects of different concentrations of *Codium fragile* extract on in vitro rumen gas production in rumen fluid incubated for 24 h are shown in Table 2. Methane concentration and production (based on mL, mL/g of dry matter, and mL/g of digested dry matter) were the lowest in the 0.5% *Codium fragile* extract treatment ($p < 0.05$).

The effects of different concentrations of *Codium fragile* extract on in vitro digestibility and rumen fermentation characteristics of rumen fluid incubated for 24 h are shown in Table 3. The concentration of butyrate was the lowest in the 0.5% *Codium fragile* extract treatment group ($p < 0.05$). The concentration of isovalerate was the highest in the 0.25% *Codium fragile* extract treatment ($p < 0.05$). In vitro dry matter digestibility and total VFAs concentration did not differ significantly among treatments ($p > 0.05$).

The effects of different concentrations of *Codium fragile* extract on in vitro rumen gas production in rumen fluid incubated for 48 h are shown in Table 4. Methane concentration and production (based on mL and mL/g of dry matter) tended to decrease in the group added with *Codium fragile* extract ($0.05 < p < 0.10$). Methane production based on digested dry matter was the lowest in the 0.5% *Codium fragile* extract treatment ($p < 0.05$).

Table 2. The effects of different levels of *Codium fragile* extract on the in vitro rumen gas production in rumen fluid incubated for 24 h.

Item	<i>Codium fragile</i> Extract Levels, %			SEM	<i>p</i> -Value
	0	0.25	0.5		
Total gas production, mL	39.2	41.0	36.3	0.636	0.205
CH ₄ concentration, % of total gas	7.60 ^a	7.19 ^{ab}	6.70 ^b	0.374	0.004
CH ₄ production, mL	2.98 ^a	2.95 ^a	2.42 ^b	0.159	0.026
CH ₄ production, mL/g of DM	5.96 ^a	5.89 ^a	4.84 ^b	0.310	0.026
CH ₄ production, mL/g of dDM	10.88 ^a	10.74 ^a	8.82 ^b	0.552	0.005

^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$. DM, dry matter; dDM, digested dry matter.

Table 3. The effects of different levels of *Codium fragile* extract on the in vitro rumen fermentation characteristics in rumen fluid incubated for 24 h.

Item	<i>Codium fragile</i> Extract Levels, %			SEM	<i>p</i> -Value
	0	0.25	0.5		
IVDMD, % of DM	54.8	54.8	54.7	0.316	1.000
pH	6.18	6.21	6.21	0.011	0.896
NH ₃ -N, mg/dL	10.46	9.26	10.63	0.420	0.375
Total VFAs, mM	58.7	57.02	63.11	1.468	0.228
Acetate, % of total VFAs	55.99	55.53	56.44	0.502	0.787
Propionate, % of total VFAs	26.56	26.34	25.3	0.391	0.398
Iso-butyrate, % of total VFAs	1.57 ^b	1.87 ^b	2.90 ^a	0.184	<0.001
Butyrate, % of total VFAs	12.37	12.51	11.82	0.136	0.085
Iso-valerate, % of total VFAs	2.59	2.79	2.62	0.056	0.325
Valerate, % of total VFAs	0.92 ^b	0.97 ^a	0.92 ^b	0.008	0.002
A:P ratio	2.11	2.11	2.26	0.057	0.494

^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$. IVDMD, in vitro dry matter digestibility; VFAs, volatile fatty acids; A:P ratio, acetate to propionate ratio.

Table 4. The effects of different levels of *Codium fragile* extract on the in vitro rumen gas production in rumen fluid incubated for 48 h.

Item	<i>Codium fragile</i> Extract Levels%			SEM	<i>p</i> -Value
	0%	0.25%	0.5%		
Total gas production, mL	69.76	67.76	64.41	0.951	0.384
CH ₄ concentration, % of total gas	8.86	8.38	7.86	0.184	0.081
CH ₄ production, mL	6.18	5.85	5.09	0.202	0.063
CH ₄ production, mL/g of DM	12.36	11.7	10.17	0.404	0.063
CH ₄ production, mL/g of dDM	17.82 ^a	17.05 ^a	13.93 ^b	0.584	0.042

^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$. DM, dry matter; dDM, digested dry matter.

The effects of different concentrations of *Codium fragile* extract on in vitro digestibility and rumen fermentation characteristics of rumen fluid incubated for 48 h are shown in Table 5. The rumen pH and NH₃-N concentration were the lowest in the 0.5% *Codium fragile* extract treatment ($p < 0.05$). The concentration of propionate was the highest in the 0.5% *Codium fragile* extract treatment ($p < 0.05$). In contrast, the concentrations of acetate, iso-butyrate, and valerate, as well as the acetate-to-propionate ratio, were the lowest ($p < 0.05$).

Table 5. The effects of different levels of *Codium fragile* extract on the in vitro rumen fermentation characteristics in rumen fluid incubated for 48 h.

Item	<i>Codium fragile</i> Extract Levels, %			SEM	<i>p</i> -Value
	0	0.25	0.5		
IVDMD, % of DM	69.33	68.59	68.82	0.334	0.080
pH	6.01 ^{ab}	6.02 ^a	6.00 ^b	0.006	0.006
NH ₃ -N, mg/dL	16.41 ^a	15.6 ^a	12.41 ^b	0.558	<0.001
Total VFAs, mM	80.53	78.59	80.10	0.760	0.586
Acetate, % of total VFAs	53.96 ^a	53.13 ^{ab}	52.95 ^b	0.177	0.029
Propionate, % of total VFAs	27.77 ^b	28.71 ^b	30.56 ^a	0.360	<0.001
Iso-butyrate, % of total VFAs	1.48	1.70	1.57	0.038	0.063
Butyrate, % of total VFAs	12.24 ^a	12.15 ^a	11.01 ^b	0.191	0.003
Iso-valerate, % of total VFAs	3.08 ^a	2.93 ^a	2.54 ^b	0.072	<0.001
Valerate, % of total VFAs	1.46	1.38	1.38	0.022	0.178
A:P ratio	1.95 ^a	1.85 ^a	1.73 ^b	0.027	<0.001

^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$. IVDMD, in vitro dry matter digestibility; VFAs, volatile fatty acids; A:P ratio, acetate to propionate ratio.

3.2. In Vivo Experiment

The effects of *Codium fragile* extract on dry matter intake, average daily gain, and methane emissions of Hanwoo steers are shown in Table 6. All diets supplied during the entire experimental period were completely consumed by all treatment groups. Methane emissions (based on day and metabolic body weight) were lower in the CFE group than in the CON group ($p < 0.05$).

Table 6. The effects of *Codium fragile* extract on dry matter intake, average daily gain, and methane emission of Hanwoo steers.

Item	Treatments		SEM	<i>p</i> -Value
	CON	CFE		
Dry matter intake, kg/day	8.50	8.50	-	-
Average daily gain, kg/day	1.61	1.51	0.260	0.786
CH ₄ emission, g/day	192.6 ^a	173.4 ^b	4.707	<0.001
CH ₄ emission, g/kg BW ^{0.75}	2.254 ^a	2.065 ^b	0.053	0.021

CON, control group; CFE, 0.5% *Codium fragile* ethanol extract. BW^{0.75}, metabolic body weight. ^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$.

The effects of *Codium fragile* extract on the rumen fermentation characteristics of Hanwoo steers are shown in Table 7. The concentration of propionate tended to increase in the CFE group compared to the CON group ($0.05 < p < 0.10$). In contrast, the concentration of butyrate and the acetate-to-propionate ratio tended to decrease ($0.05 < p < 0.10$).

Table 7. The effects of *Codium fragile* extract on rumen pH, NH₃-N, and volatile fatty acids of Hanwoo steers.

Item	Treatments		SEM	<i>p</i> -Value
	CON	CFE		
pH	6.47	6.52	0.048	0.431
Total volatile fatty acids, mM	98.0	93.0	1.960	0.215
Acetate, % of total VFAs	62.6	62.3	0.472	0.647
Propionate, % of total VFAs	19.4	20.6	0.467	0.057
Iso-butyrate, % of total VFAs	1.74	1.63	0.088	0.376
Butyrate, % of total VFAs	13.6	12.9	0.410	0.087
Iso-valerate, % of total VFAs	1.47	1.45	0.054	0.759
Valerate, % of total VFAs	1.20	1.20	0.030	0.743
Acetate:propionate ratio	3.27	3.04	0.091	0.088
NH ₃ -N, mg/dL	14.2	14.2	1.033	0.940

CON, control group; CFE, 0.5% *Codium fragile* ethanol extract.

The effects of *Codium fragile* extract on the blood metabolites of Hanwoo steers are shown in Table 8. None of the blood metabolites were affected ($p > 0.05$) by *Codium fragile* extract, except for blood urea nitrogen. Blood urea nitrogen concentration was lower in the CFE group than in the CON group ($p < 0.05$).

Table 8. The effects of *Codium fragile* extract on the blood metabolites of Hanwoo steers.

Item	Treatments		SEM	p-Value
	CON	CFE		
Glucose, mg/dL	71.9	71.6	1.328	0.852
Creatinine, mg/dL	1.31	1.33	0.794	0.670
Blood urea nitrogen, mg/dL	14.6 ^a	13.4 ^b	0.570	0.043
BUN/CREA ratio	11.3	10.0	0.417	0.053
Inorganic phosphate, mg/dL	8.15	8.26	0.163	0.561
Calcium, mg/dL	9.75	9.76	0.064	0.881
Total protein, g/dL	6.98	7.05	0.047	0.458
Albumin, g/dL	2.98	3.03	0.039	0.315
Globulin, g/dL	4.00	4.03	0.035	0.724
Albumin/Globulin ratio	0.74	0.75	0.013	0.620
Alanine aminotransferase, U/L	49.6	53.5	1.865	0.060
Alkaline phosphatase, U/L	149.1	159.0	12.65	0.233
Gamma-glutamyl transferase, U/L	18.3	18.1	0.702	0.881
Total bilirubin, mg/dL	0.33	0.36	0.018	0.168
Cholesterol, mg/dL	94.1	95.1	2.711	0.712
Amylase, U/L	36.9	40.0	1.653	0.165
Lipase, U/L	81.1	102.4	9.452	0.083

CON, control group; CFE, 0.5% *Codium fragile* ethanol extract. ^{a, b} Means without a common superscript letter differ significantly, $p < 0.05$.

4. Discussion

4.1. In Vitro Experiment

Recently, *Codium fragile* has been reported to inhibit ruminal methane production [16,17]. Furthermore, Kim et al. [9] demonstrated that the methane-mitigating effects of *Codium fragile* are associated with the content of very-long-chain and unsaturated fatty acids. Consistent with these findings, the present study also observed reduced methane production in *Codium fragile* when expressed on a digested dry matter basis (Tables 2 and 4).

In the present study, ruminal $\text{NH}_3\text{-N}$ concentrations decreased with increasing levels of *Codium fragile* extract (Tables 3 and 5). Several studies reported that an additive with unsaturated fatty acids can reduce rumen $\text{NH}_3\text{-N}$ concentrations by enhancing microbial protein synthesis during ruminal fermentation [18,19]. Similarly, the results of this study suggest that fatty acids present in *Codium fragile* extract contribute to the reduction in rumen $\text{NH}_3\text{-N}$ by promoting microbial protein synthesis. However, the chemical composition of the *Codium fragile* extract was not characterized in the present study. Therefore, the specific compounds responsible for these effects could not be definitively identified.

After 48 h of in vitro incubation, the decrease in acetate and increase in propionate observed in the 0.5% *Codium fragile* treatment resulted in a decrease in the acetate: propionate ratio. According to a previous study, an additive with unsaturated and long-chain fatty acids suppresses ruminal methane production by reducing methanogen and protozoan activities and altering fermentation pathways [20]. In addition, fatty acids have been shown to reduce methane release and methanogenic counts during rumen fermentation, and increased dietary unsaturated fatty acids have been linked to decreased abundance of methanogenic archaea, contributing to reduced methane emissions [19]. As in previous studies, the inhibition of methane production in the present study may have increased the

availability of metabolic hydrogen. Consequently, hydrogen may have been diverted from methanogenesis toward propionate-producing pathways, as evidenced by a lower acetate-to-propionate ratio in the 0.5% *Codium fragile* extract treatment. In contrast, additives with unsaturated fats have been reported to reduce ruminal protozoa and methanogenic archaea, leading to shifts in fermentation patterns and decreased methane production [21]. A decrease in ruminal protozoa has been shown to decrease butyrate and increase propionate concentrations [22,23]. The present study also showed a similar decrease in the butyrate concentration (Table 5), which may reflect both the preferential utilization of metabolic hydrogen for propionate production and the inhibitory effects of unsaturated fatty acids in *Codium fragile* on protozoa and butyrate-producing bacteria. However, further studies are required to clarify the relationship between changes in ruminal protozoan populations and shifts in volatile fatty acid profiles, particularly in butyrate concentration.

4.2. In Vivo Experiment

The inclusion of seaweed in ruminant diets has been associated with reduced palatability when provided in unprocessed form or as ground seaweed, primarily because of odor and volatile compounds [24,25]. Previous studies reported that solvent extraction, particularly ethanolic extraction, can improve palatability by selectively removing compounds responsible for odor [26]. In addition, several studies demonstrated that seaweed extract does not decrease the feed intake of ruminants [27,28]. In the present study, dry matter intake did not differ between treatments under the controlled feeding conditions used in this experiment. Methane emissions (based on day and metabolic body weight) decreased in the *Codium fragile* extract treatment. The results were consistent with those observed in the in vitro experiment, suggesting that the methane-mitigating effect of the extract may be mediated by the direct modulation of rumen microbial activity rather than secondary effects associated with changes in feed intake or animal performance.

Although significant changes in propionate, butyrate, and the acetate-to-propionate ratio were observed in the in vitro experiment, these responses were only observed in vivo ($0.05 < p < 0.10$). This discrepancy is likely attributable to biological variations among animals and the buffering capacity of the rumen environment, which can attenuate shifts in volatile fatty acid profiles in vivo [29]. Nevertheless, the consistent direction of responses between the in vitro and in vivo experiments supports the biological relevance of the effects of the extract under practical feeding conditions.

Blood urea nitrogen (BUN) concentration was significantly lower in the *Codium fragile* extract treatment group than in the control group (Table 8; 13.4 vs. 14.6 mg/dL; $p < 0.05$). BUN concentration is widely used as an indicator of hepatic urea synthesis and nitrogen utilization metabolism in ruminants [30]. In general, a reduction in BUN concentration indicates a decrease in ammonia converted into urea in the liver, which may suggest alterations in nitrogen metabolism [31]. Although urinary nitrogen excretion was not directly measured in the present study, a significant decrease in the BUN concentration may indicate reduced urea synthesis and a potential reduction in urinary urea-N loss. In addition, both values were included in the physiological normal range (10.7–18.1 mg/dL) for Hanwoo steers [32], indicating the health of steers fed a *Codium fragile* extract.

There are several limitations to consider when interpreting the results of the present study. Although the methane-mitigating effect of *Codium fragile* extract was confirmed in vitro and in vivo, the chemical composition of the extract was not analyzed. Therefore, only speculative assumptions can be made regarding the compounds responsible for the observed effects, and the specific bioactive compounds have not yet been identified. In addition, the optimal inclusion level of 0.5% was determined under controlled experimental

conditions, and further studies are needed to validate its efficacy across different feeding systems and long-term production settings.

5. Conclusions

In conclusion, the inclusion of *Codium fragile* extract at 0.5% demonstrated effective methane mitigation in cattle without negatively affecting feed intake, growth performance, or health-related blood parameters. These findings indicate that 0.5% inclusion represents an optimal level under the conditions of this study and suggest the potential of *Codium fragile* extract as a practical methane-mitigating feed additive in ruminant production systems.

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