fermentation

MDPI

Article

Evaluation of Different Brown Seaweeds as Feed and Feed Additives Regarding Rumen Fermentation and Methane Mitigation

Eslam Ahmed [1,2,*], Belgutei Batbekh [3], Naoki Fukuma [1,4], Masaaki Hanada [1] and Takehiro Nishida [1,*]

[1] Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro 080-8555, Japan
[2] Department of Animal Behavior and Management, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt
[3] Graduate School of Animal Husbandry, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro 080-8555, Japan
[4] Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro 080-8555, Japan
* Correspondence: eslam_kh@obihiro.ac.jp (E.A.); nishtake@obihiro.ac.jp (T.N.); Tel.: +81-155-49-5455 (T.N.)

Abstract: This study investigated the impacts of different brown seaweed species—*Ascophyllum nodosum*, *Sargassum fulvellum*, *Ecklonia maxima*, *Lessonia flavicans*, *Lessonia nigrescens*, and *Laminaria japonica*—on rumen fermentation and methane (CH_4) mitigation. The current in vitro batch culture study for 24 h at 39 °C evaluated these species in two experimental designs: as feed additive and as feed. The control group for both experimental designs was composed of 500 mg of basal diet (50% grass hay/50% concentrate). For the feed additives experimental design, each seaweed species was evaluated when it was added at 20% of the basal diet, while as a feed, the inclusion level of each species was 20% to partially replace the concentrate in the basal diet as follows (50% hay/30% concentrate/20% seaweed). Chemical analyses showed that the seaweeds were characterized by a high fiber content and high amounts of minerals such as calcium, potassium, and phosphorus, while the protein content ranged within 7 and 13%. When they were applied as feed additives, they increased the production of volatile fatty acids, with *L. japonica* being the most effective; however, they failed to suppress CH_4 production. In contrast, their inclusion as a feed in the basal diet led to a significant reduction ($p < 0.05$) in CH_4, especially for *E. maxima* and *L. japonica*, by up to 18 and 21%, respectively, but this was associated with general inhibition of the rumen fermentation. Therefore, the tested seaweeds could be used as a source of minerals and as a feed additive to improve rumen fermentation, but without anti-methanogenic potential. Meanwhile, their inclusion as feed at 20% could reduce CH_4 production with an adverse effect on fermentation. Thus, further trials are needed to identify the appropriate inclusion level to achieve effective CH_4 reduction without any detrimental effects on rumen fermentation.

Keywords: alternative feed; global warming; dietary manipulation; digestibility; macroalgae; sustainability

Citation: Ahmed, E.; Batbekh, B.; Fukuma, N.; Hanada, M.; Nishida, T. Evaluation of Different Brown Seaweeds as Feed and Feed Additives Regarding Rumen Fermentation and Methane Mitigation. *Fermentation* **2022**, *8*, 504. https://doi.org/10.3390/fermentation8100504

Academic Editor: Anusorn Cherdthong

Received: 11 September 2022
Accepted: 28 September 2022
Published: 30 September 2022

1. Introduction

In recent years, there has been a rising interest in the usage of seaweed in many applications [1]. Many studies have provided evidence of seaweeds as promising and sustainable feed additives/supplements for the livestock industry. They can be used as an alternative source of nutrients since they contain myriad nutrients, including proteins, lipids, carbohydrates, vitamins, and minerals [2,3]. Previous studies have reported the positive impacts of some seaweeds on animal health, performance, and product quality [4–6]. Seaweeds can be categorized into three distinct groups based on their pigmentation: brown, green, and red [5]. Although there are about 10,000 species of marine seaweeds distributed along

coastal regions worldwide, only a few of them have been evaluated as feed ingredients in ruminants' diets [7].

Besides their nutritional value, seaweeds are gaining more interest as anti-methanogenic feed ingredients since they possess bioactive compounds, such as halogenated and polyphenolic secondary metabolites, that have shown potency in reducing methane (CH_4) emissions from ruminants [8]. The red seaweed *Asparagopsis taxiformis* is rich in halogenated compounds, particularly bromoform, which has strong efficacy in reducing CH_4 production, potentially by over 80% [9,10]. However, sustainable supply of this seaweed is an issue, and there are some concerns over its production due to the potential negative environmental impacts on the ozone layer, rumen health, and bromoform's health impacts on humans [11]. Therefore, there is an active search underway to identify new efficient and safe bioactive molecules from seaweeds to be employed in climate-friendly ruminant feed. Brown seaweeds have a higher and more diversified content of bioactive compounds with high commercial interest [12]. They are the only algae to contain polyphenol phlorotannins [13]. Phlorotannins have broad-spectrum antimicrobial activity, especially on rumen cellulolytic bacteria such as *Fibrobacter succinogenes* [14]. The chemical structure of phlorotannins is similar to that of tannins from terrestrial plants [15]. Terrestrial tannins were reported to have anti-methanogenic activity, either by direct inhibition on methanogenic archaea or indirectly through inhibiting other rumen microorganisms associated with methanogenesis [16]. Therefore, the efficacy of phlorotannin-containing brown seaweeds against rumen methanogenesis may be worth further exploration. Some studies have reported successful CH_4 reduction with supplementation of some brown seaweeds or their extracts, while others observed no effect, with inconsistent effects on other rumen fermentation parameters [17–21]. The species specificity, usage, and dosage might be the reasons for discrepancies among studies. Although there are many species of brown seaweeds, very few of them have been evaluated as feed additives for ruminants [5]. Furthermore, there is a gap in the current knowledge regarding how to apply brown seaweeds in ruminants' diets either as a feed additive or as feed to replace part of the conventional ingredients [22,23]. It is noteworthy that there have been no studies conducted on the actually available brown seaweed commercial products in this respect.

Some of the seaweed species used in the current study, such as *Ascophyllum nodosum* and *Sargassum fulvellum* were evaluated previously in a few studies, with inconsistent results [18,24,25], while for others, such as *Ecklonia maxima*, *Lessonia flavicans*, *Lessonia nigrescens*, and *Laminaria japonica*, it would be the first time to be evaluated. The brown seaweed species are well known for their lower nutritional value (low protein and fat contents with higher mineral contents) while containing about 1100 bioactive compounds [4,5]. Therefore, the main objective of this study was to evaluate them as potential anti-methanogenic feed additives, as well as determining any potential unfavorable effects on rumen fermentation characteristics. Additional objective of this study was to evaluate their usage as a feed to partially replace the concentrate mixture in the diet while assessing their impacts on CH_4 and rumen fermentation parameters through a brief study. The hypothesis was that the usage of these species as feed additive would reduce CH_4 production due to phlorotannins effect without adverse effect on rumen fermentation profile. Additionally, we hypothesized that these macroalgae might be used as an alternative feed to replace part of the conventional feed ingredient (concentrate mixture) in the basal diet with the potential to reduce CH_4 production and without adverse impacts on fermentation characteristics.

2. Materials and Methods

This study was conducted in Obihiro University of Agriculture and Veterinary Medicine, Japan, following the standard experimental procedures approved by the Committee of Animal Care and Ethics (approval number, 21–212). Animals used in this study were kept and cared for by the Field Science Center, Obihiro University.

2.1. Basal Diets and Seaweeds

The basal diet was composed of ground (1 mm size) Kleingrass (*Panicum coloratum*) hay and a commercial concentrate mixture (Alpha-Kotan, Chubu Shiryo Co., Ltd., Aichi, Japan). The chemical compositions of the grass hay and concentrate are described in Table 1. Seven kinds of commercial brown seaweeds were provided by an agricultural trading company (Andes Trading Co., Ltd., Tokyo, Japan). The seaweeds used in the current study were as follows: *A. nodosum* (collected from Ireland), *A. nodosum* (collected from Canada), *E. maxima* (collected from South Africa), *S. fulvellum* (collected from Indonesia), *L. flavicans* (collected from Chile), *L. nigrescens* (collected from Chile), and *L. japonica* (collected from China). More details about the sites of collection and processing of each product are described in Table 2.

Table 1. Chemical composition of the basal diet (g/kg in dry matter) used for 24 h in vitro incubation.

g/kg	Kleingrass Hay	Concentrate Mixture
Dry matter (g/kg in fresh matter)	910	875
Organic matter	892	937
Crude ash	108	63
Crude protein	146	188
Ether extract	37	43
Neutral detergent fiber	626	315
Acid detergent fiber	339	117
Acid detergent lignin	63	26
Non-fiber carbohydrate	83	391

Table 2. Site of harvesting and processing of the evaluated commercial brown seaweed products.

Product Name	Seaweed Species	Site of Collection	Drying Method	Drying Temperature	Crushing Method
Algin gold	*Ascophyllum nodosum*	North Atlantic Sea Basin, Ireland	Rotary Kiln Dryer	60–80 °C for 1.5 h	Hammer mill
Asco Sea Green	*Ascophyllum nodosum*	North Atlantic Sea Basin, Canada	Rotary Kiln Dryer	60–80 °C for 1.5 h	Hammer mill
Ecklonia gold	*Ecklonia maxima*	South Atlantic Sea Basin (offshore of Western Cape State), South Africa	Sun Drying	-	Hammer mill
Seaweed meal	*Sargassum fulvellum*	Sunda Strait, Indonesia	Rotary Kiln Dryer	60–80 °C for 1.5 h	Hammer mill
Lessonia gold	*Lessonia flavicans*	Chilean Sea, Chile	Sun Drying	-	Hammer mill
Lessonia gold	*Lessonia nigrescens*	Chilean Sea, Chile	Sun Drying	-	Hammer mill
Laminaria gold	*Laminaria japonica*	Bohai Sea and Yellow Sea, China	Rotary Kiln Dryer	60–80 °C for 1.5 h	Hammer mill

2.2. Donor Animals and Rumen Fluid Collection

Approximately 1.3 L of rumen fluid was collected 3 h after morning feeding from two ruminally fistulated non-lactating cows approximately 8 years old with an average body weight of 894 kg. The cows were fed at the maintenance level on a diet of orchard grass (*Dactylis glomerata*) hay (organic matter (OM), 980 g/kg; crude protein (CP), 132 g/kg; neutral detergent fiber (NDF), 701 g/kg; acid detergent fiber (ADF), 354 g/kg; acid detergent lignin (ADL), 40 g/kg; dry matter (DM) basis) with free access to clean drinking water and mineral blocks (Koen® E250 TZ, Nippon Zenyaku Kogyo Co., Fukushima, Japan). The collected rumen fluid was strained through four layers of surgical gauze, placed into a Thermos flask that had been pre-warmed to 39 °C, and then immediately transferred to the laboratory within 15 min.

2.3. Experimental Design and In Vitro Incubation Procedure

This study was conducted following two different experimental designs. The first experiment (EXP. 1) was performed with a control diet (basal diet, 50% hay/50% concentrate), and each seaweed species was used as feed additive at 20% of the substrate. The second experiment (EXP. 2) was performed with the control group (EXP. 1), and each seaweed species was included in the basal diet at a rate of 20%, replacing part of the concentrate

mixture (50% hay/30% concentrate/20% seaweed). Each treatment in both experimental designs had four replicates, and two bottles were used as blanks. EXP. 1 was repeated in three separate runs in different weeks, while EXP. 2 was performed in one experimental run, representing a small-scale preliminary study to provide us with some insights on the usage of these macroalgae as alternative feed.

Approximately 500 mg of substrate was added to pre-weighed nylon bags with a pore size of 53 ± 10 µm (BG1020, Sanshin Industrial Co., Ltd., Kanagawa, Japan), which were heat-sealed and placed in 120 mL glass bottles. In case of EXP. 1, seaweed feed additives were added directly to the bottles. Under continuous carbon dioxide (CO_2) flushing, 40 mL of fresh buffer solution (pH 6.8) [26] and 20 mL of the collected rumen fluid were added to each fermentation bottle. The bottles were then flushed with CO_2 before being sealed with butyl rubber stoppers and aluminum caps (Maruemu Co., Ltd., Osaka, Japan). All the bottles were incubated for 24 h at 39 °C.

2.4. Incubation Medium Sampling and Analysis

At the end of the incubation, the total gas production was measured using calibrated syringe and an aliquot of the headspace gas was collected from each bottle and stored in a vacutainer tube (BD Vacutainer, Becton Drive, NJ, USA) until CH_4 and CO_2 were determined by gas chromatography (GC-8A, Shimadzu Corp., Kyoto, Japan), as described previously by Ahmed et al. [27]. Then, the pH was determined, and 1 mL of the culture medium was collected in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and centrifuged at 16,000× *g* at 4 °C for 5 min. The supernatant was used to estimate the volatile fatty acid (VFA) content via high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) as processed and previously described [27]. The nylon bags were rinsed with tap water until the effluent became clear, after which they were dried at 60 °C for 48 h and weighed to determine the in vitro dry matter digestibility (IVDMD). The IVDMD was calculated as the DM that disappeared from the initial DM weight input into the bag.

2.5. Chemical Analysis

The chemical composition analyses of the grass hay and concentrate mixture were performed at Obihiro University according to AOAC standard procedures [28]. The DM content was determined by drying the samples in an air-forced oven at 135 °C for 2 h (method 930.15). The OM was measured by placing the samples in a muffle furnace at 500 °C for 3 h (method 942.05). The ether extract (EE) was determined according to method 920.39, while nitrogen was measured according to the Kjeldahl method (method 984.13) using an electrical heating digester (DK 20, VELP Scientifica, Usmate (MB), Italy) and an automatic distillation apparatus (UDK 129 VELP Scientifica, Usmate (MB), Italy). The CP was then estimated as nitrogen × 6.25. NDF, ADF, and ADL were estimated and expressed as inclusive residual ash using an ANKOM^{200} fiber analyzer (Ankom Technology Methods 6, 5, and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). NDF was measured using sodium sulfite without the heat-stable α-amylase. The chemical compositions of the seaweeds were determined by Tokachi Federation of Agricultural Cooperative Associations, Obihiro, Japan, according to AOAC standard procedures [28]. A Foss fibertec™ 8000 fiber analysis system (FOSS, Hilleroed, Denmark) was used to determine the NDF, ADF, and ADL. The minerals except phosphorus were determined using an atomic absorption spectrophotometer (AA-7000, Shimadzu Corporation, Kyoto, Japan). Phosphorus was determined via the vanadomolybdate colorimetric method (QuAAtro 39; BL TEC K. K., Osaka, Japan). The non-fiber carbohydrate (NFC) was estimated according to the following formula: NFC g/kg = 1000 − (NDF g/kg + CP g/kg + EE g/kg + Ash g/kg). The total digestible nutrients (TDN) of the seaweeds were calculated using the formula proposed by NRC [29]. The chemical compositions of the brown seaweeds are described in Table 3.

Table 3. Chemical composition (g/kg in dry matter) of the brown seaweeds used for 24 h in vitro incubation.

g/kg	*A. nodosum* (Ireland)	*A. nodosum* (Canada)	*E. maxima* (South Africa)	*S. fulvellum* (Indonesia)	*L. flavicans* (Chile)	*L. nigrescens* (Chile)	*L. japonica* (China)
Dry matter (g/kg in fresh matter)	873	881	877	853	907	930	905
Organic matter	705	708	582	680	506	324	602
Crude ash	295	292	418	320	494	676	398
Crude protein	76	89	116	95	101	79	133
Ether extract	22	32	4.0	9.0	5.0	5.0	6.0
Neutral detergent fiber	431	315	392	457	265	200	298
Acid detergent fiber	273	219	232	231	157	109	275
Acid detergent lignin	207	101	93	136	74	69	38
Non-fiber carbohydrate	228	330	148	187	184	78	254
Total digestible nutrients	263	408	238	256	232	86	337
Ca	12	16	42	36	42	96	778
P	1.0	1.6	3.1	1.2	17	9.7	2.1
Mg	8.3	10	11	12	13	13	5.8
K	26	23	43	83	81	55	35

2.6. Statistical Analysis

All data were analyzed using SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC, USA). Data from EXP. 1 were analyzed using PROC MIXED with models including the treatments as a fixed effect, whereas the three experimental runs were considered random effects. For EXP. 2, data were analyzed via one-way analysis of variance (ANOVA) using the PROC GLM. For all experiments, the values are shown as means with pooled standard errors of the means. Differences in means among the experimental groups were estimated using Tukey's test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. EXP. 1

Adding 20% of brown seaweeds as a feed additive to the basal diet increased the yield (mL/g) of total gas production, CH_4, and CO_2 per digestible DM (D.DM) when compared with the control group ($p < 0.01$, Table 4). Similarly, production of total VFA increased ($p < 0.01$) with the supplementation of all seaweeds except *L. nigrescens* (Table 5). Notably, adding *L. japonica* resulted in the highest concentration of total VFA when compared with the control group or even with other seaweed species ($p < 0.05$). The IVDMD was not changed by the supplementation of seaweeds ($p > 0.05$, Table 5).

3.2. EXP. 2

Inclusion of the brown seaweeds at a rate of 20%, partially replacing the concentrate mixture in the basal diet, had no effect on the yield (mL/g) of total gas or CO_2/D.DM ($p > 0.05$, Table 6). *A. nodosum*, *S. fulvellum*, *L. flavicans*, and *L. nigrescens* slightly decreased the yield (mL/g) of CH_4/D.DM by 9.6, 9.5, 4.8, and 12.6%, respectively, when compared with the basal diet without seaweeds, but this was not significant ($p > 0.05$, Table 6). The inclusion of *E. maxima* and *L. japonica* in the basal diet significantly reduced the yield of CH_4/D.DM by 18.3% ($p = 0.017$) and 21.1% ($p = 0.005$), respectively, when compared with the control group (Table 6). The IVDMD was significantly lower ($p < 0.05$) with the inclusion of the seaweeds in the control diet, except with *A. nodosum* harvested from Ireland, *S. fulvellum*, and *L. flavicans*, for which the IVDMD was comparable to that in the control group ($p > 0.05$, Table 7). Additionally, the production of total VFA decreased significantly with the inclusion of all tested seaweeds ($p < 0.01$, Table 7). The fermentation profile was shifted toward more acetate and less propionate with 20% inclusion of seaweeds in the basal diet ($p < 0.01$, Table 7).

Table 4. Effect of different brown seaweeds as feed additives on gas production profile from 24 h in vitro incubation (n = 12) (Experiment 1).

Parameter	Control	*A. nodosum* (Ireland)	*A. nodosum* (Canada)	*E. maxima* (South Africa)	*S. fulvellum* (Indonesia)	*L. flavicans* (Chile)	*L. nigrescens* (Chile)	*L. japonica* (China)	SEM	*p* Value
Total Gas/DM [1] (mL/g)	102.71 ^{c}	116.09 ^{b}	115.64 ^{b}	114.01 ^{b}	116.93 ^{b}	116.78 ^{b}	111.14 ^{bc}	132.75 ^{a}	1.51	<0.001
Total gas/D.DM [2] (mL/g)	176.06 ^{c}	192.8 ^{b}	194.59 ^{b}	195.94 ^{b}	191.5 ^{b}	189.4 ^{b}	187.1 ^{b}	216.76 ^{a}	1.64	<0.001
CH_4 (%)	5.83 ^{b}	6.02 ^{ab}	5.87 ^{b}	5.77 ^{b}	5.95 ^{b}	6.13 ^{ab}	5.91 ^{b}	6.37 ^{a}	0.05	<0.001
CO_2 (%)	94.16 ^{a}	93.97 ^{ab}	94.12 ^{a}	94.22 ^{a}	94.04 ^{a}	93.86 ^{ab}	94.08 ^{a}	93.62 ^{b}	0.05	<0.001
CH_4/DM (mL/g)	6.03 ^{c}	7.03 ^{b}	6.84 ^{bc}	6.62 ^{bc}	7.01 ^{b}	7.21 ^{b}	6.61 ^{bc}	8.49 ^{a}	0.14	<0.001
CH_4/D.DM (mL/g)	10.27 ^{c}	11.62 ^{b}	11.45 ^{b}	11.32 ^{b}	11.40 ^{b}	11.62 ^{b}	11.07 ^{bc}	13.83 ^{a}	0.16	<0.001
CO_2/DM (mL/g)	96.67 ^{c}	109.05 ^{b}	108.8 ^{b}	107.39 ^{b}	109.77 ^{b}	109.57 ^{b}	104.53 ^{bc}	124.26 ^{a}	1.38	<0.001
CO_2/D.DM (mL/g)	165.79 ^{c}	181.17 ^{b}	183.14 ^{b}	184.62 ^{b}	180.06 ^{b}	177.78 ^{b}	176.03 ^{b}	202.92 ^{a}	1.50	<0.001

[1] DM, Dry matter. [2] D.DM, Digestible dry matter. SEM: Standard error of the mean. $^{a, b, c}$ Values with different superscripts in the same row are significant different ($p < 0.05$).

Table 5. Effect of different brown seaweeds as feed additives on rumen fermentation characteristics from 24 h in vitro incubation (n = 12) (Experiment 1).

Parameter	Control	*A. nodosum* (Ireland)	*A. nodosum* (Canada)	*E. maxima* (South Africa)	*S. fulvellum* (Indonesia)	*L. flavicans* (Chile)	*L. nigrescens* (Chile)	*L. japonica* (China)	SEM	*p* Value
pH	6.58 ^{a}	6.56 ^{ab}	6.56 ^{abc}	6.54 ^{bc}	6.52 ^{bcd}	6.52 ^{cd}	6.53 ^{bcd}	6.50 ^{d}	0.01	<0.001
IVDMD [1] (%)	58.37	60.29	59.31	58.20	60.99	61.65	59.50	61.33	0.58	0.100
Acetate (mmol/L)	347.55 ^{c}	360.63 ^{b}	360.26 ^{b}	364.48 ^{ab}	364.37 ^{ab}	363.60 ^{ab}	358.01 ^{bc}	374.38 ^{a}	9.75	<0.001
Propionate (mmol/L)	82.74 ^{c}	87.35 ^{ab}	88.93 ^{ab}	88.14 ^{ab}	89.36 ^{b}	88.28 ^{b}	86.10 ^{bc}	91.44 ^{a}	2.51	<0.001
Butyrate (mmol/L)	24.47 ^{b}	25.19 ^{ab}	24.58 ^{b}	24.59 ^{b}	25.53 ^{ab}	25.25 ^{ab}	24.88 ^{ab}	26.25 ^{a}	0.88	0.003
Total VFA [2] (mmol/L)	454.77 ^{c}	473.17 ^{b}	473.78 ^{b}	477.23 ^{ab}	479.27 ^{ab}	477.13 ^{ab}	469.00 ^{bc}	492.08 ^{a}	13.03	<0.001
Acetate (mol/100mol)	76.66	76.44	76.23	76.54	76.18	76.42	76.58	76.23	0.16	0.094
Propionate (mol/100mol)	18.08 ^{b}	18.38 ^{ab}	18.71 ^{a}	18.41 ^{ab}	18.62 ^{ab}	18.43 ^{ab}	18.25 ^{ab}	18.53 ^{ab}	0.13	0.016
Butyrate (mol/100mol)	5.24 ^{a}	5.17 ^{abc}	5.04 ^{c}	5.03 ^{c}	5.19 ^{abc}	5.14 ^{abc}	5.15 ^{abc}	5.22 ^{ab}	0.06	0.001

[1] IVDMD: In vitro dry matter digestibility. [2] VFA: Volatile fatty acids. SEM: Standard error of the mean. $^{a, b, c, d}$ Values with different superscripts in the same row are significant different ($p < 0.05$).

Table 6. Effect of different brown seaweeds as feed on gas production profile from 24 h in vitro incubation (n = 4) (Experiment 2).

Parameter	Control	*A. nodosum* (Ireland)	*A. nodosum* (Canada)	*E. maxima* (South Africa)	*S. fulvellum* (Indonesia)	*L. flavicans* (Chile)	*L. nigrescens* (Chile)	*L. japonica* (China)	SEM	*p* Value
Total Gas/DM [1] (mL/g)	101.92 [a]	91.08 [ab]	88.73 [b]	87.13 [b]	93.89 [ab]	94.64 [ab]	88.15 [b]	87.87 [b]	1.17	0.008
Total gas/D.DM [2] (mL/g)	165.78	162.45	166.34	161.80	156.56	160.88	164.39	159.77	1.42	0.75
CH_4 (%)	5.92 [a]	5.45 [abc]	5.36 [abc]	4.97 [bc]	5.66 [ab]	5.81 [a]	5.22 [bc]	4.84 [c]	0.08	<0.001
CO_2 (%)	94.08 [c]	94.55 [abc]	94.64 [abc]	95.03 [ab]	94.34 [bc]	94.19 [bc]	94.78 [ab]	95.16 [a]	0.08	<0.001
CH_4/DM (mL/g)	6.04 [a]	4.97 [abc]	4.76 [bc]	4.33 [bc]	5.35 [abc]	5.50 [ab]	4.60 [b]	4.26 [bc]	0.13	0.001
CH_4/D.DM (mL/g)	9.82 [a]	8.85 [ab]	8.91 [ab]	8.02 [b]	8.88 [ab]	9.35 [a]	8.58 [ab]	7.75 [b]	0.15	0.005
CO_2/DM (mL/g)	95.87 [a]	86.11 [ab]	83.97 [b]	82.79 [b]	88.54 [ab]	89.14 [ab]	83.55 [b]	83.61 [b]	1.05	0.012
CO_2/D.DM (mL/g)	155.96	153.59	157.43	153.77	147.68	151.53	155.81	152.02	1.34	0.756

[1] DM, Dry matter. [2] D.DM, Digestible dry matter. SEM: Standard error of the mean. [a, b, c] Values with different superscripts in the same row are significant different ($p < 0.05$).

Table 7. Effect of different brown seaweeds as feed on rumen fermentation characteristics from 24 h in vitro incubation (n = 4) (Experiment 2).

Parameter	Control	*A. nodosum* (Ireland)	*A. nodosum* (Canada)	*E. maxima* (South Africa)	*S. fulvellum* (Indonesia)	*L. flavicans* (Chile)	*L. nigrescens* (Chile)	*L. japonica* (China)	SEM	*p* Value
pH	6.65	6.75	6.74	6.68	6.69	6.72	6.68	6.67	0.01	0.132
IVDMD [1] (%)	61.44 [a]	56.09 [abc]	53.42 [bc]	54.09 [bc]	59.83 [a]	58.86 [abc]	53.60 [c]	55.05 [bc]	0.66	0.001
Acetate (mmol/L)	202.60 [a]	191.89 [bc]	189.18 [bc]	186.40 [bc]	192.71 [abc]	195.80 [ab]	187.30 [bc]	184.85 [c]	1.18	<0.001
Propionate (mmol/L)	45.48 [a]	38.05 [b]	37.99 [b]	37.00 [b]	38.07 [b]	39.40 [b]	37.13 [b]	38.03 [b]	0.51	<0.001
Butyrate (mmol/L)	15.12 [a]	13.27 [b]	12.87 [b]	13.29 [b]	13.73 [ab]	13.96 [ab]	13.26 [b]	13.49 [b]	0.15	0.001
Total VFA [2] (mmol/L)	263.19 [a]	243.21 [b]	240.05 [b]	236.70 [b]	244.50 [b]	249.16 [b]	237.69 [b]	236.37 [b]	1.75	<0.001
Acetate (mol/100mol)	76.98 [b]	78.90 [a]	78.81 [a]	78.75 [a]	78.83 [a]	78.58 [a]	78.80 [a]	78.20 [a]	0.12	<0.001
Propionate (mol/100mol)	17.28 [a]	15.65 [b]	15.83 [b]	15.63 [b]	15.56 [b]	15.82 [b]	15.62 [b]	16.09 [b]	0.11	<0.001
Butyrate (mol/100mol)	5.74 [a]	5.45 [ab]	5.36 [b]	5.62 [ab]	5.61 [ab]	5.60 [ab]	5.58 [ab]	5.71 [ab]	0.03	0.036

[1] IVDMD: In vitro dry matter digestibility. [2] VFA: Volatile fatty acids. SEM: Standard error of the mean. [a, b, c] Values with different superscripts in the same row are significant different ($p < 0.05$).

4. Discussion

The current study evaluated the potency of seven species of brown seaweeds as feed and feed additives supplemented to a ruminant diet at 20%. This higher dosage level was used for feed additives to ensure that the tested species could show their mode of action, since the design of this study was short-term in vitro batch culture for only 24 h. It is important to mention that some of the differences in the results might be interpreted by the fact that when the seaweeds were used as additives, their experimental treatments had an extra 100 mg (600 mg substrate) versus 500 mg substrate in the control basal diet. Although the EXP. 2 as a preliminary study provided some new insights on the usage of these macroalgae as alternative feed, it had a shortcoming of being conducted with only one experimental run. Therefore, the results from that design should be interpreted with caution.

Seaweeds are a very heterogeneous group of nutrients, and their application in the ruminant feeding industry has been restricted due to scarce information on their nutritive value. The present study provides the nutritional value of some new species which have not been evaluated so far. The current study confirmed what was reported previously that the ash content in seaweeds is very high and can range between 200 g/kg and 700 g/kg of DM [25,30,31]. Generally, the brown seaweed species are characterized by higher amounts of ash and minerals than green and red species [30,32]. The macroalgae evaluated in the present study were rich in some essential minerals, especially Ca and K. According to Pino and Heinrichs [33], these higher amounts of minerals could be helpful for ruminants in stimulating rumen microbes and fermentation. Therefore, they can be considered as a source of minerals to maintain ruminants' health and prevent some mineral deficiencies. In addition, the tested seaweeds can be considered good sources of fiber (NDF and ADF), as described previously by Lahaye [34]; thus, they can be used as a prebiotic feed additive to enhance the health status of both monogastric and ruminant animals. In contrast, the proximate analysis showed that the tested brown seaweeds had low levels of CP and EE. The highest amount of CP belonged to *L. japonica*, with a value near to that of grass hay, while its EE content was less than 10 g/kg. It was reported that the CP contents in most brown seaweed species are less than 150 g/kg, while the fat content is classified within 10–50 g/kg [2,5,35,36]. It is important to mention that the concentration of nutrients in seaweeds is highly variable across seasons and geographical locations [37–39], which was observed in this study with *A. nodosum* harvested in Ireland and Canada.

The rumen fermentation characteristics responded differently to the way in which seaweeds were applied. Using brown seaweed species as feed additives improved the fermentation rate through increasing the production of total VFA and CO_2, the main indicators of the fermentation rate [40]. This means that adding seaweeds could have stimulated the activity of rumen microbes to utilize the seaweeds as a feed/prebiotic. This finding also could have arisen due to the extra 100 mg of seaweed added as an additive, which must be taken into account when interpreting this result. The variation in the production of total VFA among the different macroalgae might be related to the nutritive value and the ease of degradability of each species; this was obvious with *L. japonica*, *S. fulvellum*, and *L. flavicans*, where their addition led to the highest concentrations of total VFA, in order. Similarly, in a recent study, the addition of *S. fulvellum* extract to the basal diet at a level of 5% of the substrate improved the production of total VFA in a batch culture for 24 h [41]. To the best of our knowledge, there is still very limited information on the impacts of these seaweeds as feed additives on the rumen fermentation profile. Conversely, the inclusion of seaweeds in the basal diet at a rate of 20% to replace part of the concentrate mixture led to a decrease in the production of total VFA and a lower IVDMD. This finding could be attributed to the lower digestibility of the tested species. This theory could be supported by what was reported previously—that the rumen degradability of most brown seaweed species has been observed to be low [5,17,42]. However, interestingly, when *S. fulvellum* was included in the diet, the IVDMD was comparable to that of the control. This was also observed in some studies in which it was reported that some

brown seaweed species, including *S. fulvellum*, had a higher OM degradability [5,43]. Marín et al. [24] reported that the inclusion of *Sargassum* spp. at up to 30% in the diet of goats had no effect on diet digestibility, but the production of total VFA tended to decrease. Therefore, the digestibility of brown seaweeds is species-dependent. Another theory for the inhibited fermentation while using brown seaweeds is related to their higher content of phlorotannins. Phlorotannins extracted from *A. nodosum* and supplemented to different diets were found to reduce in vitro rumen fermentation through decreasing the activity and abundance of bacterial groups involved in fiber degradability, such as *Fibrobacter succinogenes* [14,21,44]. The mode of action of phlorotannins was observed to affect the integrity of the microbial cell membrane and cell wall, as well as the inactivation of extracellular enzymes and proteins necessary for the growth and metabolism of rumen microorganisms [45,46]. Furthermore, another factor that could have a major effect on rumen fermentation and should be considered is the inclusion level of macroalgae in the diet [47]. According to a review study, seaweeds cannot be used as a complete substitute for typical animal feed, and their inclusion level should not be more than 10% of the animal feed; otherwise, they will have negative effects [23]. Therefore, further research is required to ascertain the optimal inclusion level of these promising species so that animal productivity is not adversely impacted.

The CH_4 reduction potential of the brown seaweed species was not detected when they were applied as feed additives. In contrast, when those species were used as feed, a decrease in CH_4 production was observed. This reduction in CH_4 might be attributed to the inhibited fermentation and the low nutrient degradability, which means diminished substrates available for methanogens to utilize for CH_4 production. There is some support for this theory in a recent study in which two species of brown seaweeds, *A. nodosum* and *Fucus vesiculosus*, were included with the basal diet at a level of 5%. Reductions in CH_4 by 8.9 and 3.6%, respectively, were associated with reductions in total gas production, IVDMD, and the production of VFA [48]. This could imply that the brown seaweeds tested in the current study might not have a direct effect on methanogenic archaea when compared with some other feed additives that are well known for their anti-methanogenic potential, such as *A. taxiformis* [49], 3-nitrooxypropanol (Bovaer®) [50], and a garlic–citrus extract (Mootral Ruminant®) [51]. Instead, the potential of brown seaweeds appeared through general inhibition of rumen fermentation and rumen microbes due to the lower availability of fermentable substrate. The strongest CH_4 reduction potential in the current study was observed with the inclusion of *L. japonica* and *E. maxima*, showing reductions of up to 21 and 18%, respectively, which were also accompanied by the lowest IVDMD and concentration of total VFA. Although the nutritive value of *L. japonica* was higher than that in most of the other tested species, it demonstrated a greater adverse effect, which might be related to its high content of phlorotannins or other bioactive compounds. This study is the first to report the efficacy of this new macroalga on rumen fermentation and CH_4 production. Therefore, further research is required to identify the secondary metabolites of this seaweed and their impacts on rumen fermentation. Generally speaking, for all the tested species, it is unclear yet whether the CH_4 reduction potential could be related to the less digestible nutrients in the seaweeds or due to the effect of the bioactive compounds, which needs further investigation.

5. Conclusions

The current study provides information on the impacts of seven brown seaweed species when they were used as feed or feed additives. Despite failing to show CH_4 reduction potential when these species were applied as feed additives, their addition at 20% of the substrate led to an increase in the production of total VFA. On the contrary, when these macroalgae were used as feed at an inclusion level of 20% in the basal diet to partially replace the concentrate mixture, they demonstrated a reduction in CH_4 yield, especially for *E. maxima* and *L. japonica*, with 18 and 21% reductions, respectively. However, this was associated with adverse effects on rumen fermentation characteristics. Therefore, these two

promising species could be evaluated in further solid research with lower inclusion rates as well as to replace grass instead of concentrates to overcome the negative impacts on rumen fermentation and also to evaluate their potency in CH_4 reduction.

Author Contributions: E.A.: Conceptualization, project administration, methodology, formal analysis, investigation, and writing—original draft. B.B.: Investigation, review and editing. T.N.: Project administration, resources, supervision, review and editing. N.F. and M.H.: Resources, review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by Andes Trading Co., Ltd., Tokyo, Japan, grant number (09102021).

Institutional Review Board Statement: The experimental procedures of this study were approved by animal care and ethics committee at the Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number, 21–212).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting reported results in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare that they have no competing interest. The funder had no role in the design of the study; in the interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Pradhan, B.; Bhuyan, P.P.; Patra, S.; Nayak, R.; Behera, P.K.; Behera, C.; Behera, A.K.; Ki, J.-S.; Jena, M. Beneficial effects of seaweeds and seaweed-derived bioactive compounds: Current evidence and future prospective. *Biocatal. Agric. Biotechnol.* **2022**, *39*, 102242. [CrossRef]
2. Øverland, M.; Mydland, L.T.; Skrede, A. Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. *J. Sci. Food Agric.* **2019**, *99*, 13–24. [CrossRef] [PubMed]
3. Rajauria, G. Seaweeds: A sustainable feed source for livestock and aquaculture. In *Seaweed Sustainability*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 389–420, ISBN 9780124186972.
4. Rjiba-Ktita, S.; Chermiti, A.; Bodas, R.; France, J.; López, S. Aquatic plants and macroalgae as potential feed ingredients in ruminant diets. *J. Appl. Phycol.* **2017**, *29*, 449–458. [CrossRef]
5. Makkar, H.P.; Tran, G.; Heuzé, V.; Giger-Reverdin, S.; Lessire, M.; Lebas, F.; Ankers, P. Seaweeds for livestock diets: A review. *Anim. Feed Sci. Technol.* **2016**, *212*, 1–17. [CrossRef]
6. Evans, F.D.; Critchley, A.T. Seaweeds for animal production use. *J. Appl. Phycol.* **2014**, *26*, 891–899. [CrossRef]
7. Guiry, M.D. The Seaweed Site: Information on Marine Algae. Available online: https://www.seaweed.ie/ (accessed on 11 September 2022).
8. Pandey, D.; Mansouryar, M.; Novoa-Garrido, M.; Næss, G.; Kiron, V.; Hansen, H.H.; Nielsen, M.O.; Khanal, P. Nutritional and anti-methanogenic potentials of macroalgae for ruminants. In *Seaweed and Microalgae as Alternative Sources of Protein*; Lei, X.G., Ed.; Burleigh Dodds Science Publishing: Cambridge, UK, 2021; pp. 195–228, ISBN 9781786766205.
9. Roque, B.M.; Venegas, M.; Kinley, R.D.; de Nys, R.; Duarte, T.L.; Yang, X.; Kebreab, E. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. *PLoS ONE* **2021**, *16*, e0247820. [CrossRef] [PubMed]
10. Machado, L.; Magnusson, M.; Paul, N.A.; Kinley, R.; de Nys, R.; Tomkins, N. Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in vitro. *J. Appl. Phycol.* **2016**, *28*, 3117–3126. [CrossRef]
11. Min, B.R.; Parker, D.; Brauer, D.; Waldrip, H.; Lockard, C.; Hales, K.; Akbay, A.; Augyte, S. The role of seaweed as a potential dietary supplementation for enteric methane mitigation in ruminants: Challenges and opportunities. *Anim. Nutr.* **2021**, *7*, 1371–1387. [CrossRef]
12. Mišurcová, L. Chemical Composition of Seaweeds. In *Handbook of Marine Macroalgae*; Kim, S.-K., Ed.; Wiley: Hoboken, NJ, USA, 2011; pp. 171–192, ISBN 9780470979181.
13. Holdt, S.L.; Kraan, S. Bioactive compounds in seaweed: Functional food applications and legislation. *J. Appl. Phycol.* **2011**, *23*, 543–597. [CrossRef]
14. Wang, Y.; Alexander, T.W.; McAllister, T.A. In vitro effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on rumen bacterial populations and fermentation. *J. Sci. Food Agric.* **2009**, *89*, 2252–2260. [CrossRef]
15. Arnold, T.M.; Targett, N.M. Marine tannins: The importance of a mechanistic framework for predicting ecological roles. *J. Chem. Ecol.* **2002**, *28*, 1919–1934. [CrossRef] [PubMed]
16. Hassan, F.-U.; Arshad, M.A.; Ebeid, H.M.; Rehman, M.S.-U.; Khan, M.S.; Shahid, S.; Yang, C. Phytogenic additives can modulate rumen microbiome to mediate fermentation kinetics and methanogenesis through exploiting diet-microbe interaction. *Front. Vet. Sci.* **2020**, *7*, 575801. [CrossRef] [PubMed]

17. Bikker, P.; Stokvis, L.; van Krimpen, M.M.; van Wikselaar, P.G.; Cone, J.W. Evaluation of seaweeds from marine waters in Northwestern Europe for application in animal nutrition. *Anim. Feed Sci. Technol.* **2020**, *263*, 114460. [CrossRef]
18. Belanche, A.; Jones, E.; Parveen, I.; Newbold, C.J. A Metagenomics approach to evaluate the impact of dietary supplementation with *Ascophyllum nodosum* or *Laminaria digitata* on rumen function in rusitec fermenters. *Front. Microbiol.* **2016**, *7*, 299. [CrossRef] [PubMed]
19. Maia, M.R.G.; Fonseca, A.J.M.; Oliveira, H.M.; Mendonça, C.; Cabrita, A.R.J. The potential role of seaweeds in the natural manipulation of rumen fermentation and methane production. *Sci. Rep.* **2016**, *6*, 32321. [CrossRef]
20. De La Moneda, A.; Carro, M.D.; Weisbjerg, M.R.; Roleda, M.Y.; Lind, V.; Novoa-Garrido, M.; Molina-Alcaide, E. Variability and potential of seaweeds as ingredients of ruminant diets: An in vitro study. *Animals* **2019**, *9*, 851. [CrossRef]
21. Wang, Y.; Xu, Z.; Bach, S.J.; McAllister, T.A. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on in vitro ruminal digestion of mixed forage or barley grain. *Anim. Feed Sci. Technol.* **2008**, *145*, 375–395. [CrossRef]
22. Erickson, P.S.; Marston, S.P.; Gemmel, M.; Deming, J.; Cabral, R.G.; Murphy, M.R.; Marden, J.I. Short communication: Kelp taste preferences by dairy calves. *J. Dairy Sci.* **2012**, *95*, 856–858. [CrossRef]
23. Morais, T.; Inácio, A.; Coutinho, T.; Ministro, M.; Cotas, J.; Pereira, L.; Bahcevandziev, K. Seaweed potential in the animal feed: A Review. *J. Mar. Sci. Eng.* **2020**, *8*, 559. [CrossRef]
24. Marín, A.; Casas-Valdez, M.; Carrillo, S.; Hernández, H.; Monroy, A.; Sanginés, L.; Pérez-Gil, F. The marine algae *Sargassum* spp. (*Sargassaceae*) as feed for sheep in tropical and subtropical regions. *Rev. Biol. Trop.* **2009**, *57*, 1271–1281. [CrossRef]
25. Choi, Y.Y.; Lee, S.J.; Kim, H.S.; Eom, J.S.; Kim, D.H.; Lee, S.S. The potential nutritive value of *Sargassum fulvellum* as a feed ingredient for ruminants. *Algal Res.* **2020**, *45*, 101761. [CrossRef]
26. McDougall, E.I. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* **1948**, *43*, 99–109. [CrossRef] [PubMed]
27. Ahmed, E.; Fukuma, N.; Hanada, M.; Nishida, T. The efficacy of plant-based bioactives supplementation to different proportion of concentrate diets on methane production and rumen fermentation characteristics in vitro. *Animals* **2021**, *11*, 1029. [CrossRef] [PubMed]
28. AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 1995.
29. National Research Council. *Nutrient Requirements of Dairy Cattle*, 7th ed.; National Academies Press: Washington, DC, USA, 2001; ISBN 978-0-309-06997-7.
30. Cabrita, A.R.J.; Maia, M.R.G.; Oliveira, H.M.; Sousa-Pinto, I.; Almeida, A.A.; Pinto, E.; Fonseca, A.J.M. Tracing seaweeds as mineral sources for farm-animals. *J. Appl. Phycol.* **2016**, *28*, 3135–3150. [CrossRef]
31. D'Armas, H.; Jaramillo, C.; D'Armas, M.; Echavarría, A.; Valverde, P. Proximate composition of several green, brown and red seaweeds from the coast of Ecuador. *Rev. Biol. Trop.* **2019**, *67*, 61–68. [CrossRef]
32. Fernández-Segovia, I.; Lerma-García, M.J.; Fuentes, A.; Barat, J.M. Characterization of spanish powdered seaweeds: Composition, antioxidant capacity and technological properties. *Food Res. Int.* **2018**, *111*, 212–219. [CrossRef]
33. Pino, F.; Heinrichs, A.J. Effect of trace minerals and starch on digestibility and rumen fermentation in diets for dairy heifers. *J. Dairy Sci.* **2016**, *99*, 2797–2810. [CrossRef]
34. Lahaye, M. Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some 'sea vegetables'. *J. Sci. Food Agric.* **1991**, *54*, 587–594. [CrossRef]
35. Biancarosa, I.; Espe, M.; Bruckner, C.G.; Heesch, S.; Liland, N.; Waagbø, R.; Torstensen, B.; Lock, E.J. Amino acid composition, protein content, and nitrogen-to-protein conversion factors of 21 seaweed species from Norwegian waters. *J. Appl. Phycol.* **2017**, *29*, 1001–1009. [CrossRef]
36. Dawczynski, C.; Schubert, R.; Jahreis, G. Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chem.* **2007**, *103*, 891–899. [CrossRef]
37. Schiener, P.; Black, K.D.; Stanley, M.S.; Green, D.H. The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta*. *J. Appl. Phycol.* **2015**, *27*, 363–373. [CrossRef]
38. Paiva, L.; Lima, E.; Neto, A.I.; Baptista, J. Seasonal variability of the biochemical composition and antioxidant properties of *Fucus spiralis* at two Azorean islands. *Mar. Drugs* **2018**, *16*, 248. [CrossRef] [PubMed]
39. Britton, D.; Schmid, M.; Revill, A.T.; Virtue, P.; Nichols, P.D.; Hurd, C.L.; Mundy, C.N. Seasonal and site-specific variation in the nutritional quality of temperate seaweed assemblages: Implications for grazing invertebrates and the commercial exploitation of seaweeds. *J. Appl. Phycol.* **2021**, *33*, 603–616. [CrossRef]
40. Owens, F.N.; Basalan, M. Ruminal Fermentation. In *Rumenology*; Millen, D.D., de Beni Arrigoni, M., Lauritano Pacheco, R.D., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 63–102, ISBN 978-3-319-30531-8.
41. Choi, Y.; Lee, S.J.; Kim, H.S.; Eom, J.S.; Jo, S.U.; Le Guan, L.; Seo, J.; Kim, H.; Lee, S.S.; Lee, S.S. Effects of seaweed extracts on in vitro rumen fermentation characteristics, methane production, and microbial abundance. *Sci. Rep.* **2021**, *11*, 24092. [CrossRef]
42. Lee, S.J.; Jeong, J.S.; Shin, N.H.; Lee, S.K.; Kim, H.S.; Eom, J.S.; Lee, S.S. Impact of *Ecklonia stolonifera* extract on in vitro ruminal fermentation characteristics, methanogenesis, and microbial populations. *Asian Australas. J. Anim. Sci.* **2019**, *32*, 1864–1872. [CrossRef]
43. Hansen, H.; Hector, B.; Feldmann, J. A qualitative and quantitative evaluation of the seaweed diet of North Ronaldsay sheep. *Anim. Feed Sci. Technol.* **2003**, *105*, 21–28. [CrossRef]

44. Targett, N.M.; Arnold, T.M. Minireview-predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *J. Phycol.* **1998**, *34*, 195–205. [CrossRef]
45. Hierholtzer, A.; Chatellard, L.; Kierans, M.; Akunna, J.C.; Collier, P.J. The impact and mode of action of phenolic compounds extracted from brown seaweed on mixed anaerobic microbial cultures. *J. Appl. Microbiol.* **2013**, *114*, 964–973. [CrossRef]
46. Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875–3883. [CrossRef]
47. Machado, L.; Magnusson, M.; Paul, N.A.; Kinley, R.; de Nys, R.; Tomkins, N. Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro fermentation and methane production. *J. Appl. Phycol.* **2016**, *28*, 1443–1452. [CrossRef]
48. Künzel, S.; Yergaliyev, T.; Wild, K.J.; Philippi, H.; Petursdottir, A.H.; Gunnlaugsdottir, H.; Reynolds, C.K.; Humphries, D.J.; Camarinha-Silva, A.; Rodehutscord, M. Methane reduction potential of brown seaweeds and their influence on nutrient degradation and microbiota composition in a rumen simulation technique. *Front. Microbiol.* **2022**, *13*, 889618. [CrossRef] [PubMed]
49. Brooke, C.G.; Roque, B.M.; Shaw, C.; Najafi, N.; Gonzalez, M.; Pfefferlen, A.; de Anda, V.; Ginsburg, D.W.; Harden, M.C.; Nuzhdin, S.V.; et al. Methane reduction potential of two pacific coast macroalgae during in vitro ruminant fermentation. *Front. Mar. Sci.* **2020**, *7*, 561. [CrossRef]
50. Romero-Pérez, A.; Okine, E.K.; Guan, L.L.; Duval, S.M.; Kindermann, M.; Beauchemin, K.A. Rapid Communication: Evaluation of methane inhibitor 3-nitrooxypropanol and monensin in a high-grain diet using the rumen simulation technique (Rusitec)1,2. *J. Anim. Sci.* **2017**, *95*, 4072–4077. [CrossRef] [PubMed]
51. Ahmed, E.; Yano, R.; Fujimori, M.; Kand, D.; Hanada, M.; Nishida, T.; Fukuma, N. Impacts of Mootral on methane production, rumen fermentation, and microbial community in an in vitro study. *Front. Vet. Sci.* **2020**, *7*, 623817. [CrossRef] [PubMed]