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The effect of the dried *Gracilaria* spp. undergoing different drying methods on *in vitro* rumen fermentation

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Abstract: Seaweed has an important role in the mitigation of enteric methane (CH_4) production by ruminant animals. The utilisation and its effectiveness in enteric CH_4 reduction require a preservation process. The aim of the study was to investigate the effect of different drying processes on the effectiveness of seaweed *Gracilaria* spp. in reducing CH_4 production assessed through an *in vitro* method. Three drying techniques, sun-drying, oven-drying, and freeze-drying, were applied to produce a dried product of *Glacilaria* spp. Rice straw basal diets combined with concentrate at the 70 : 30% were used to test the inclusion of 4% of three differently dried products of *Gracilaria* spp. compared to the basal diet without seaweed (control group). Measurements were conducted on *in vitro* total and CH_4 gas production, nutrient degradability, ammonia (NH_3) and VFA concentration, and microbial population. Results showed that the three dried products of *Gracilaria* spp. significantly reduced *in vitro* CH_4 production compared to the control group ($P \leq 0.05$). All drying techniques gave a similar effect on *in vitro* CH_4 reduction, but they did not affect dry matter (DM) and organic matter (OM) degradability ($P \leq 0.05$) and significantly reduced neutral detergent fiber (NDF) degradability ($P \leq 0.05$) compared to the control group, with oven-dried treatments having the lowest NDF degradability among the treatments. It is concluded that the three different drying techniques had similar effects on enteric CH_4 reduction.

Keywords: enteric methane; methane production; mitigation; preservation; seaweed

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Ruminant animals play an important role in fulfilling the protein demand in the world. On the other hand, they also contribute greatly to the world's greenhouse gas emissions (GHG). Enteric methane (CH_4) is the major GHG released by ruminants, which accounts for about 90% of the total emissions from ruminants or about 40% of the total emissions from livestock (Doyle et al. 2019). Many methods for decreasing enteric CH_4 emissions have been introduced in recent decades. The emphasis is mostly laid on animal nutrition, including feed additives, feed supplements, and balance of the nutrients in the diet. Increasing the legume leaf proportion in the diet as a feed supplement (Vijn et al. 2020) and using seaweed as a dietary feed supplement lead to a decrease in CH_4 production in the rumen (Widiawati and Hikmawan 2021).

Three families of seaweeds, including brown (Phaeophyceae), green (Chlorophyta) and red (Rhodophyta) seaweed, have shown reduced enteric CH_4 production (Vijn et al. 2020; Cheong et al. 2023). Red seaweeds such as *Asparagopsis taxiformis* produce a variety of halogenated secondary compounds like bioactive bromoform, some of which are potential candidates for exerting an anti-methanogenic effect (Mihaila et al. 2022; Nilsson and Martin 2022). Moreover, a study conducted in Indonesia suggests that certain types of seaweed mainly cultivated along the coast on many islands in Indonesia like *Eucheuma cottonii* have a potential to reduce enteric CH_4 production (Widiawati and Hikmawan 2021). Another type of seaweed which is mostly cultivated along the coast in the western part of Indonesia is *Gracilaria* spp.

Fresh seaweed has a different content of moisture in the range from 69.41% up to 86.33% (Kustantinah et al. 2022). Using fresh seaweed as an ingredient in the feed industry will bring some problems related to the time period and the technical storage as well as the quality stability. Also, feedstuffs that contain moisture higher than 70% should not be ensiled due to the potential seepage losses and growth of undesirable bacteria (such as clostridia) during the ensilage process, which results in undesirable fermentation. Thus, the best moisture content of the feedstuff used for ruminant feed is up to 30%. Therefore, drying seaweed has become one of the solutions to reduce the moisture content of the seaweed to be used as feed for ruminants.

The effect of three different drying techniques, namely sun-drying, oven-drying, and freeze-drying, was tested on *Sargassum* spp. by Paga et al. (2022), who found that the effectiveness of bioactive compounds in the seaweed to mitigate CH_4 gas production was not affected by the three different drying processes, although another study showed that drying seaweed reduced beneficial phenolic compounds in the seaweed (Silva et al. 2019). The use of seaweed as an ingredient in the food industry often requires it to be dried before use (Gupta and Abu-Ghannam 2011). A drying process helps prevent decomposition, increases shelf life and aids the extraction of certain chemical constituents contained in the seaweeds. Previously, three various drying techniques, such as sun-drying, freeze-drying, and oven-drying, were used in the food industry.

Various species of seaweed have been tested to reduce enteric CH_4 gas from ruminants (Muizelaar et al. 2022; Thorsteinsson et al. 2023). Each species of seaweed has its different bioactive content, which also has a different impact on decreasing the enteric CH_4 gas production. The red seaweed is one of the potential marine resources whose production is abundant but it has not been widely utilised in Indonesia (Kustantinah et al. 2022). One type of red seaweed is *Gracilaria* spp. that is easy to cultivate with a low production cost. The production is continuous because it can be harvested regularly (every 40–50 days). This seaweed is resistant to the effects of fresh water, and can even live in brackish water. Many types of *Gracilaria* pp. are found on the beaches of large islands. However, only a few studies have tested the effectiveness of the seaweed *Gracilaria* spp. in reducing enteric CH_4 emissions. There is also limited information on the best preservation techniques for *Gracilaria* spp. without affecting its effectiveness in lowering the enteric CH_4 production. Therefore, the study aim was to investigate the effect of the drying process of *Gracilaria spp.* and its potential to reduce *in vitro* CH_4 production.

MATERIALS AND METHODS

Seaweed collection and preparation

The seaweed *Gracilaria* spp. was collected from the Lontar village in Serang City, Banten, Tangerang, Indonesia. *Gracilaria* spp. was dried in three ways: oven-dried, sun-dried, and freeze-dried.

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For an oven-dried sample, the seaweed was dried in an oven at 60 °C for 2–3 days or until it reached the moisture content of about 15–20%. For a sun-dried sample, the seaweed was kept under the sun's natural light for 3–4 days; between 8 a.m. to 4 p.m. for 4 to 5 days with the ambient temperature range of 29–33 °C. The drying process was stopped when the moisture content of seaweed was about 15–20%. While for a freeze-dried sample, the seaweed was stored and dried in the freeze dryer at –80 °C for about 6–7 days. The sun-dried, freeze-dried, and oven-dried samples were then ground to form a fine powder and filtered by using a 1 mm sieve and they were stored in the freezer for further use.

Donor animals

The ruminally cannulated Ongole crossbred steers were used in the study as sources of rumen inoculum. They were cared for according to the guidelines of the Research Centre for Animal Husbandry, National Research and Innovation Agency (BRIN). The study was conducted under the Animal Ethics (No.: 193/KE.02/SK/10/2023) released by the Commission of Ethics for Maintenance and Animal Use for Research, National Research and Innovation Agency.

The cattle have free access to drinking water every day. The diet consisted of fresh pachong grass and concentrate (70 : 30; %). The nutrient content of the feed used is presented in Table 1.

The nine-kg dry weight of daily ration was divided into two equal amounts offered twice a day: at around 8 a.m. and at 2 p.m. The nutrient content of the ration on a dry matter (DM) basis was estimated to fulfil the requirement for the maintenance of steers as recommended by Nutrient Requirements in Beef Cattle Nutrition Series (2018), including crude protein 11.7%, total digestible nutrients (TDN) 63.7%, net energy for maintenance (NEm) 0.335 Mcal/kg, Ca 0.49% and P 0.24%.

Table 1. The nutrient content of the feeds offered to ruminally cannulated Ongole crossbred steers

Type of feed	Nutrients content			
	CP (%)	CF (%)	EE (%)	GE (call/g)
Pakchong grass	7.52	38.22	2.87	4 371
Concentrate	11.43	18.40	3.03	3 849

CF = crude fibre; CP = crude protein; EE = ether extract; GE = gross energy

Source: proximate analysis at BRIN Laboratorium

The whole rumen content from each of the steers was collected 1 h before the morning feeding time. The contents were mixed using a blender for about 3 min; after 3 min, the mixture was filtered through two layers of cheesecloth to collect the filtrate to be used for inoculum. The inoculum was kept in Erlenmeyer flasks placed in water bath at 39 °C under CO_2 gas flushing to keep the inoculum in an anaerobic condition.

In vitro incubation

In vitro incubation was conducted using two methods: using gas-tight culture bottles according to Theodorou et al. (1994) to collect CH_4 and total gas production and to determine DM and organic matter (OM) digestibility in the rumen, NH_3 and volatile fatty acids (VFA) production, and microbial population. The second method was an ANKOM Daisy II incubator (Ankom Technology, USA) to determine the NDF digestibility in the rumen. A total mixed ration (TMR) consisting of Pachong grass and concentrate at a ratio of 70 : 30 was used as a basal diet for the *in vitro* incubation.

The seaweed poowwder samples were added to the TMR folling four treatments: namely control (TMR + no seaweed); oven-dried (TMR 0.5 g + oven-dried seaweed powder 0.02 g); sun-dried (TMR 0.5 g + sun-dried seaweed powder 0.02 g); and freeze-dried (TMR 0.5 g + freeze-dried seaweed powder 0.02 g). Each treatment was replicated 5 times, and two bottles without TMR were used as blanks. In this study, McDougall's buffer was used to support the growth of rumen bacteria with a composition of sodium bicarbonate ($NaHCO_3$) 9.8 g/l, anhydrous sodium hydrogen phosphate (Na_2HPO_4) 3.71 g/l, potassium chloride (KCl) 0.57 g/l, sodium chloride (NaCl) 0.47 g/l, (magnesium sulfate ($MgSO_4$) heptahydrate 0.12 g/l, dihydrate calcium chloride ($CaCl_2$) 0.05 g/l and urea 0.9 g/l (Camacho et al. 2019). The ratio between McDougall's buffer and rumen fluid was 2 : 1.

During the incubation process, the gas was measured at 24 h and 48 hours. Total gas produced was measured using glass syringes equipped with a 21G needle (Terumo Indonesia, Jakarta, Indonesia) inserted into the empty part of bottles. The gas volume

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collected in the syringes was recorded as the total gas produced. Then, the total gas collected was inserted into a 10 ml vacuum vial bottle using a needle equipped with three-way taps. During the incubation time, all vial bottles filled with gas were kept in the freezer for further analysis of CH_4 concentration.

After 48 h of incubation, a liquid sample from each bottle was transferred into distinguishing centrifuge tubes and then centrifuged at 3 000 rpm for 10 minutes (Low speed-LC-8S; JoanLab, Huzhou, China). The centrifuge process was repeated twice. Two sets of 22 microtubes were filled with approximately 5 ml of the filtrate liquid from each bottle. Each set of microtubes were further used for determination of NH_3, VFAs, bacteria, and protozoa population produced during the 48 h of incubation time. The centrifuge tubes with the residues were then kept in the oven at 60 °C for 72 h until they were completely dry to be used to determine DM degradability in the rumen.

The second *in vitro* method was performed using the ANKOM Daisy II incubator device to determine the rumen digestibility of OM and NDF following the procedure of Guney (2019). Sixty-four (n = 64) F-57 filter bags were filled with 4 feed treatments (n = 16/treatment), 14 bag replicates for each treatment, plus 2 blanks of each treatment. The feed treatments used for the ANKOM Daisy II incubator were: control (TMR + no seaweed); oven-dried (TMR 0.5 g + oven-dried seaweed powder 0.02 g); sun-dried (TMR 0.5 g + sun-dried seaweed powder 0.02 g); and freeze-dried (TMR 0.5 g + freeze-dried seaweed powder 0.02 g). After 48 h, the jars were removed out of the incubator. The liquid from each jar was discarded. The bags from each jar were soaked in acetone for 5 min and then stored in the freezer for further analysis for NDF and OM.

Analysis of parameters

The CH_4 concentration of the samples was analysed using gas chromatography (GC) (GC-14 B; Shimadzu, Kyoto, Japan). Samples for NH_3 concentration analysis previously stored in microtubes were analysed using the Conway microdiffusion technique (Conway 1962; Park and Lee 2020). Samples for VFA concentration were analysed using gas chromatography (Chrompack CP-9002) for total and partial VFA concentrations. The microbial population, referred to as the number of protozoa, was determined using a haemocytometer, and the bacterial population was recorded by the roll tube method (Ogimoto and Imai 1981).

Statistical analysis

Collected data were analysed by one-way analysis of variance (ANOVA) using the compare means model procedure of SPSS 25.0 to obtain mean and standard error of the mean (SEM).

Differences between treatments ($P < 0.05$) were tested using the Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Total gas production and composition

Data on *in vitro* total gas production, CH_4 production, and CH_4 per total gas produced (%) among the treatments are described in Table 2. The CH_4 production demonstrated the total amount of CH_4 produced during 48 h of incubation. Meanwhile, CH_4 per total gas produced (%) provides the percentage value of CH_4 produced relative to the total gas production within each treatment. In general, dried seaweeds increased total gas produced and degradability but reduced the proportion of CH_4 produced. However, different dried seaweeds (oven-dried, sun-dried, and freeze-dried) have a varying effect on rumen fermentation kinetics as indicated by parameters measured in this experiment.

After 48 h of incubation time, the freeze-dried and sun-dried treatments have significantly higher total gas production, then followed by oven-dried and control treatments ($P \leq 0.01$). The amount of total gas production in the control group was similar to that of the oven-dried group. The total gas production was similar between sun-dried and freeze-dried groups. The study also showed that all dried seaweeds (oven-dried, sun-dried, and freeze-dried) significantly reduced CH_4 production and CH_4 per total gas produced (%) ($P \leq 0.01$). All the dried seaweeds (oven-dried, sun-dried, and freeze-dried) showed a similar effect in reducing CH_4 produced during 48 hours of incubation.

The CH_4 produced in the rumen represents a loss of dietary energy to the host ruminant. The higher CH_4 production indicates more energy lost,

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Table 2. Total gas and CH_4 produced during 48 h of incubation of feed supplemented with oven-dried, sun-dried, or freeze-dried seaweed *Gracilaria* spp. tested using the *in vitro* method

Parameters	Teatments				SEM	*P*-value
	control (n = 5)	oven-dried (n = 5)	sun-dried (n = 5)	freeze-dried (n = 5)		
TGP (ml)	64.4[a]	66.2[ab]	70[ab]	72.4[b]	1.015	≤ 0.01
CH_4 production (ml)	4.78	3.58[a]	3.83[a]	3.48[a]	0.211	≤ 0.05
CH_4 per TGP (%)	7.42[b]	5.40[ba]	5.47[ba]	4.79[a]	0.334	≤ 0.05
DMD (g)	0.16[a]	0.17[ab]	0.18[ab]	0.19[b]	0.004	≤ 0.05
OMD (g)	0.18[a]	0.20[a]	0.21[a]	0.21[a]	0.004	≤ 0.05
TGP/g DMD (ml/g)	388	387	374	379	6.610	≥ 0.05
TGP/g OMD (ml/g)	347	323	329	339	6.382	≥ 0.05
CH_4 produced/g DMD (ml/g)	28.8[b]	21.0[ba]	20.5[ba]	18.5[a]	1.415	≤ 0.05
CH_4 produced/g OMD (ml/g)	25.6[b]	17.5[ba]	18.1[ba]	16.5[a]	1.266	≤ 0.05

[a,b]Different superscripts in the same row indicate a significant difference

DMD = dry matter degraded; OMD = organic matter degraded; SEM = standard error of the mean; TGP = total gas produced

which means lower feed efficiency and utilisation. As reported by Sondakh et al. (2012), enteric CH_4 production, as a by-product of feed fermentation in the rumen, shows a negative correlation with the energy efficiency in ruminants. The CH_4 production represents a loss of around 8% of the total digestible energy of a feed and is released as a greenhouse gas emitted into the environment (Morgavi et al. 2008). The conversion of feed material to CH_4 in the rumen involves the integrated activities of different microbial species, with the final step carried out by methanogenic bacteria (Moss et al. 2000).

The finding of the present study was coherent with the result of Chagas et al. (2019), who indicated that dried seaweed *Asparagopsis taxiformis* reduced enteric CH_4 production when it was offered at the level of 0.05% of feed DM. Moreover, when the *A. taxiformis* was added up to 2% of feed OM, the CH_4 production was reduced by 99% (Machado et al. 2018). This supplementation was believed to be equivalent to the halogenated CH_4 analogue bromoform of 5 mM. In the present study, there was a variation in the reduction of CH_4 produced by 21.79, 30.35 and 26.46% when 4% of oven-dried, sun-dried and freeze-dried seaweed *Gracilaria* spp. on a DM basis was added, respectively. Some metabolic by-products contained in the seaweed may also affect methanogenesis. Besides the bromoform, *Gracilaria* spp. contain some bioactive compounds, including terpenoids, phenolics, flavonoids, and alkaloids (Julyasih 2022).

The *in vitro* CH_4 production when the three dried seaweeds were added to the feed was significantly lower compared to the control group ($P \leq 0.01$). However, among the three dried-seaweed treatment groups, the addition of sun-dried seaweed resulted in the lowest CH_4 production per gram of degraded OM. This result indicated the effectiveness of the sun-drying process compared to oven-drying and freeze-drying processes in terms of the effectiveness in reducing CH_4 production.

Feed degradability

The three dried seaweeds of *Gracilaria* spp. influenced the DM, OM and NDF degradability (Figure 1). The degradability of dietary DM and OM when oven-dried seaweed was added was similar to the DM and OM degradability of feed in the control group. While the DM and OM degradabilities of feed when oven-dried seaweed was added were similar to the degradabilities when sun-dried and freeze-dried seaweeds were added ($P \geq 0.05$). However, the DM and OM degradabilities in sun-dried and freeze-dried seaweed groups were significantly different from those of the control group. However, the result of the study showed that there was no significant effect of adding three dried seaweeds on NDF degradability.

When the total gas produced was calculated per unit of degraded DM and OM, the addition of three different dried seaweeds (oven-dried, sun-dried, and

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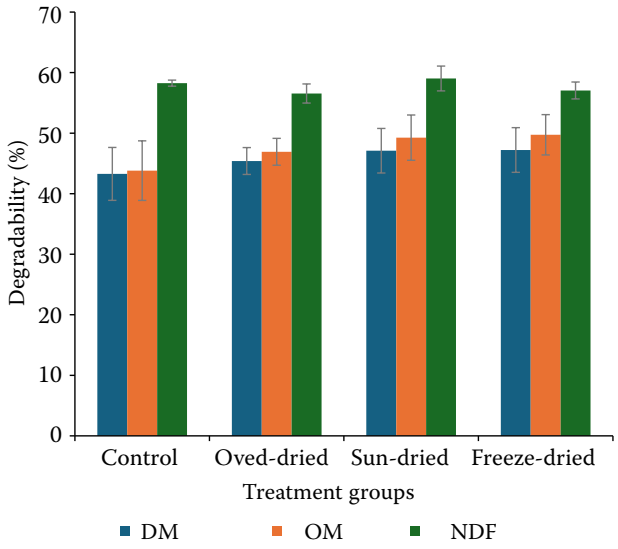

Figure 1. Degradability of DM, OM and NDF of feed supplemented with dried *Gracilaria* spp.
DM = dry matter; OM = organic matter; NDF = neutral detergent fiber

freeze-dried) resulted in a similar amount of total gas produced per gram of DM and OM ($P \geq 0.05$) (Table 2). However, when the CH_4 production was calculated per unit of degraded DM and OM, the addition of three different dried seaweeds (oven-dried, sun-dried, and freeze-dried) resulted in significantly lower CH_4 production compared to the control group ($P \leq 0.01$). Among the three treatment groups, the amount of CH_4 produced per gram of degraded DM was similar.

Seaweed contains secondary metabolites such as tannin, bromoform and iodomethane which are destroyed by drying in the sun. Secondary metabolites can function as antimicrobials, which can affect DM and OM degradability because their degradability is carried out by rumen microbes and enzymes produced by rumen microbes. Freeze-drying and oven-drying are thought to be able to maintain the content of secondary metabolites, thus affecting the viability of microbes and, in turn, affecting the degradability of DM and OM. Moreover, the study by Widiawati et al. (2024) indicated that the sun-drying process did not reduce the metabolite constituents such as total phenolics, alkaloids, flavonoids, and saponins contained in the seaweed *Euchaema cottonii*. The DM and OM degradability values in this study are in line with the percentage of CH_4 gas per total gas produced. The results of the present study were similar to the researches conducted by Dong et al. (2022).

End-product of rumen fermentation kinetics

Results of the effect of supplementation of a certain powder of the seaweed *Gracilaria* spp. produced using three different drying techniques on the end-product of rumen fermentation kinetics are presented in Table 3. The drying technique of *Gracilaria* spp. affects VFA production. The oven-drying technique reduced the total VFA production significantly and resulted in the lowest concentration among the treatments ($P \leq 0.05$). The sun-drying and freeze-drying techniques had no negative effect on the production of total VFA during feed fermentation in the rumen. Our research data showed that the sun-dried treatments tend to produce the highest amount of VFA concentration, followed by freeze-dried, control, and oven-dried treatments in a decreasing order. The seaweed contains many minerals, vitamins, phenols, polysaccharides, sterols and other bioactive substances which can influence the composition and viability of bacteria in the rumen (El-Beltagi et al. 2022). The heating method is thought to influence the availability of bioactive substances in a *Gracilaria* extract which have an impact on the composition and viability of rumen bacteria that play a role in the fermentation of feed organic materials into VFA (Guo et al. 2020; Zhang et al. 2023). The combination of dietary, microbial, environmental, and animal-related factors interacts to influence the rumen fermentation and VFA production, the factors that influence the production and composition of VFA in the rumen (Gleason et al. 2022).

The three main products of VFAs inside the rumen are C_2, C_3, and C_4. The ratio of $C_2 : C_3$ provides crucial data as it informs the researchers about the efficacy and function of feed supplementation administered to the ruminants. It provides an insight to understand the type and efficiency of fermentation occurring within the rumen. In line with the total VFA concentration, oven-drying also significantly reduced the concentration of C_2 as well as the ratio between C_2 and C_3 ($P \leq 0.05$). However, the three drying techniques did not affect the production of C_3 and C_4 ($P \geq 0.05$). On the other hand, the percentage of C_3 and C_4 in total VFAs displays the alternative results in which their highest amount is present in oven-dried samples and the lowest amount is present in sun-dried samples, as shown in Table 3. Notably, sun-dried and

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Table 3. The end-product of rumen fermentation kinetics of feed supplemented with oven-dried, sun-dried, or freeze-dried seaweed *Gracilaria* spp. during 48 h of incubation time using an *in vitro* method

Parameters	Treatments				SEM	*P*-value
	Control (n = 5)	Oven-dried (n = 5)	Sun-dried (n = 5)	Freeze-dried (n = 5)		
Total VFA concentration (mM)	125.09^{b}	98.07^{a}	135.03^{b}	130.16^{b}	5.290	≤ 0.05
C_2 concentration (mM)	107^{b}	77.9^{a}	119^{b}	114^{b}	5.664	≤ 0.05
C_3 concentration (mM)	11.4	10.8	10.2	10.9	0.408	≥ 0.05
C_4 concentration (mM)	4.85	5.75	3.89	4.42	0.291	≥ 0.05
Ratio C_2 : C_3	9.39^{ab}	7.33^{a}	11.9^{b}	10.6^{b}	0.626	≤ 0.05
Percentage C_2 from total VFA	85.6^{b}	79.6^{a}	88.4^{b}	87.8^{b}	1.178	≤ 0.01
Percentage C_3 from total VFA	9.13^{ab}	10.9 ^{b}	7.57^{a}	8.33^{a}	0.461	≤ 0.05
Percentage C_4 from total VFA	3.92^{a}	5.83^{b}	2.90^{a}	3.39^{a}	0.374	≤ 0.01
VFA/DMD (mM)/g	20.8^{a}	17.4^{a}	25.2^{b}	25.4^{b}	1.145	≤ 0.01
VFA/OMD (mM/g)	23.6^{a}	20.4^{a}	29.3^{b}	28.3^{b}	1.210	≤ 0.01
NH_3 concentration (mM)	2.80	2.80	2.80	2.80	0.000	≥ 0.05
Bacteria population ($\times 10^9$) cell/ml	4.25^{c}	3.51^{b}	2.51^{a}	3.53^{b}	0.159	≤ 0.01
Protozoa population ($\times 10^5$) cell/ml	22.5^{a}	25.0^{a}	29.8^{b}	23.4^{a}	0.876	≤ 0.01

$^{a–c}$Different superscripts in the same row indicate a significant difference

C_2 = acetic acid; C_3 = propionic acid; C_4 = butyric acid; DMD = dry matter degraded; OMD = organic matter degraded; SEM = standard error of the mean; VFA = volatile fatty acids

freeze-dried treatments tend to produce high volatile fatty acids (VFAs) per unit of degraded DM and OM, while control and oven-dried treatments seem to produce low amounts.

The NH_3 concentration in this study remains consistent in each drying method, as shown in Table 3. The *P*-value ($P \geq 0.05$) suggests that the drying techniques of seaweed did not affect protein degradation in the rumen since NH_3 in the rumen resulted from dietary protein degradation in the rumen. Data generated from the present study showed that the 4% addition of the seaweed *Gracilaria* spp. reduced the rumen bacterial population significantly ($P \leq 0.01$). Decreases in the bacterial population as a result of *Gracilaria* spp., addition are in line with a reduction in CH_4 production. Data generated from the current experiment showed that the drying process of seaweed significantly reduced the rumen bacterial population ($P \leq 0.01$). The lowest bacterial population was found in the sun-dried treatment. At the same time, the protozoa population was only affected by the sun-drying technique, which showed an increase in the protozoa population compared to other treatments. This result is different from the results shown by other studies which show that a decrease in CH_4 production will be in line with a decrease in protozoa population. Further testing is required to determine the cause of this difference in results.

Data generated from the present study showed that the 4% addition of the seaweed *Gracilaria* spp. reduced the bacterial population significantly, which is in line with a reduction in CH_4 production. The findings of the present study are similar to the findings of Machado et al. (2018), who revealed a relationship between the decline in CH_4 production and the decline in the relative abundance of methanogenic bacteria, which are part of the total bacterial population. The drying process of seaweed significantly reduced the rumen bacterial population, with the lowest bacterial population being in the sun-dried treatment.

The findings of the present study indicated a variation in the effect of the addition of each dried seaweed *Gracilaria* spp. (treatments) on the rumen fermentation kinetics. The protozoa population is the highest in the sun-drying technique compared to other treatments. At the same time, freeze-drying is the best technique in terms of reducing CH_4 gas production (Table 2), DM degradation (Figure 1) and the total bacterial population (Table 3). However, sun-drying techniques resulted

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in the best total VFA production (Table 3) and the lower amount of CH_4 gas produced from each unit of degraded DM. This may be caused by the different techniques of the drying process (oven-dried, sun-dried, and freeze-dried). There was an interaction effect between the drying process and the type of seaweed in terms of the quality and function of chemical and bioactive compounds (Silva et al. 2019). The bioactive and secondary compound effectiveness is varied depending on the drying process and also on the type of seaweed/algae. The study by Bolek (2020) showed that when the algae *Cystoseira barbata* were oven-dried at 80 °C, the chemical composition and antioxidant activity were affected. This may have occurred due to the Maillard reactions and caramelisation that resulted from the high temperature of the drying process (above 40 °C). The Maillard reaction and caramelisation contributed to a reduction in the antioxidant activity. Moreover, freeze-drying is the most appropriate drying process to maintain the effectiveness of flavonoid, carotenoid, and anthocyanin contents. However, based on the results of the previous study it can be seen that among the three drying techniques, namely oven-drying, sun-drying and freeze-drying, the sun-drying technique is the most effective for the seaweed *Gracilaria* spp. in terms of a reduction of the CH_4 production per unit of degraded OM. It is necessary to choose one of the simpler and more economical drying techniques when considering the large-scale or commercial production of dried seaweed for application as feed additive to ruminant animals.

CONCLUSION

Based on the study, it can be concluded that the sun-dried seaweed of *Gracilaria* spp. is the most effective in a reduction of the *in vitro* CH_4 production. The 4% addition of the sun-dried seaweed of *Gracilaria* spp. to the Pachong grass diet mixed with 30% concentrate reduced the *in vitro* CH_4 production up to 30.35%. The results of the study indicated that the sun-drying technique is the best to preserve the seaweed *Gracilaria* spp.

Conflict of interest

The authors declare no conflict of interest.

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