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Reducing methane production by rumen modulation through tropical seaweed supplementation in Napier grass

Redução da produção de metano por meio da modulação ruminal com suplementação de algas tropicais em capim-Napier

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ABSTRACT

Seaweed has been proven as reducing agent of ruminant methane emissions as well as increasing ruminant productivity. The present study evaluated the effect of supplementing three types of tropical seaweeds at different levels to Napier grass (*Pennisetum purpureum cv.* Gama Umami=GU) based on methane gas production and *in vitro* rumen fermentation parameters. When *P. perforate* was supplemented on GU at 5.00% organic matter (OM), the highest methane reduction was shown at 24 and 48 hour incubation (83.8% and 74.4%, respectively, P<0.01). Supplementation of 5.00% OM *P. perforate* on GU also resulted in the lowest (P<0.01) protozoa population (3.83×10^4/mL). However, adding *P. perforate* to GU lowers total gas production (a+b fraction) from 62.7 to 52.8 mL/200 mg DM, P<0.01). In terms of rumen fermentation parameters, *P. perforate* supplementation increased (P<0.01) total short-chain fatty acids (SCFA), as well as decreased acetate:propionate ratio (P<0.05) without negatively affecting rumen pH. Although ammonia (NH_3) concentration was not affected, significant rumen synthesis microbial protein increases were noted. Supplementation with *E. compressa* 2.50% OM and *C. linum* (O.F.Mull) Kutz 7.50% OM increased methane in 24 hours incubation at 12.72% and 48 hours incubation at 6.08%. Supplementation of *P. perforate* at 5.00% OM on GU decreased methane production as well as increasing total SCFA and rumen microbial protein. *P. perforate* (Bory) K.W.Nam can be used as a potential agent of methane reduction in ruminants to enhance their production.

Index terms: Decreasing methane; feed additive; macroalga; secondary metabolite.

RESUMO

As algas marinhas tem sido consideradas como um agente redutor das emissões de carbono de ruminantes bem como de aumentar a produtividade de ruminantes. O presente estudo avalia o efeito da suplementação de três tipos de algas tropicais em diferentes níveis para o capim-Napier (*Pennisetum purpureum* cv. Gama Umami=GU) com base na produção de gás metano e da fermentação ruminal *in vitro*. Quando *Palisada perforate* foi suplementado em GU a 5,00% de matéria orgânica (MO), a maior redução de metano foi observada em 24 e 48 horas de incubação (83,8% e 74,4%, respectivamente, P < 0,01). A suplementação de 5,00% de OM *P. perforate* em GU também resultou na menor (P<0,01) população de protozoários ($3,83\times10^4$/mL). Adicionar *P. perforate* a GU reduz a produção total de gás (fração a+b) de 62,7 para 52,8 mL/200 mg MS, P<0,01). Em termos de parâmetros de fermentação ruminal, a suplementação de *P. perforate* aumentou (P<0,01) os ácidos graxos de cadeia curta (AGCC) total, bem como diminuiu a razão acetato:propionato (P<0,05) sem afetar negativamente o pH do rúmen. Embora a amônia (NH_3) não tenha sido afetada, aumentos significativos na proteína microbiana de síntese ruminal foram observados. A suplementação com *Enteromorpha compressa* 2,5% OM e *Chaetomorpha linum* (O.F.Mull) Kutz 7,5% OM aumentou o metano em 24 horas de incubação em 12,72% e 48 horas de incubação em 6,08%, respectivamente. A suplementação de *P. perforate* a 5,00% de OM em GU diminuiu a produção de metano, bem como aumentou o SCFA total e a proteína microbiana ruminal. *P. perforate (Bory)* K.W.Nam pode ser usado como um agente potencial de redução de metano em ruminantes para aumentar sua produção.

Termos para indexação: Diminuição de metano; aditivo alimentar; macroalga; metabólito secundário.

Introduction

Livestock is well known as one of the contributors to greenhouse gas (GHG) emissions, which correlate with climate change. The livestock sector is responsible for 17.0% of total anthropogenic GHG emissions from enteric methane fermentation (Pandey et al., 2021). In Indonesia, a report by Widiawati and Rofiq (2019) indicates that the largest contributors of enteric methane fermentation of livestock were beef cattle (18.0 $GgCO_2$-eq/year; 74.8%), followed by goats 2.04 $GgCO_2$-eq/year (8.47%), sheep 1.51 $GgCO_2$-eq/year (6.26%), buffalo 1.40 $GgCO_2$-eq/year (5.79%), dairy cattle 0.97 $GgCO_2$-eq/year (4.00%), and horses 0.17 $GgCO_2$-eq/year (0.69%). Several strategies for reducing enteric methane production have been investigated, including the addition of ionophores, chemicals, legumes, essential oils, fats, probiotics, and secondary

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metabolites in plants (Min et al., 2021). Honan et al. (2021) stated that using seaweed as an additive is the most effective enteric methane reduction strategy. Seaweed supplementation in diet has been proven can reduce methane emissions and increase the productivity of ruminants (Roque et al., 2019; Kinley et al., 2020; Min et al., 2021; Choi et al., 2021). De Bhowmick and Hayes (2023) stated the content of seaweed bioactive compounds (polyphenols, alkaloids, saponins, halocarbons, one of which is bromoform), fats (especially unsaturated fatty acids), and complex carbohydrates, contribute to reducing methane production, both directly and indirectly. The direct mechanism is reducing populations or enzyme activity of methanogenic archaea and protozoa and indirectly modifying the rumen environment to shift the metabolic pathway of methanogenesis.

Indonesia is the second largest seaweed producing country in the world (38.7%) after China (47.9%) (Food and Agriculture Organization - FAO, 2018) and has the largest species diversity in the world (Erniati et al., 2016). Indonesia exports seaweed in 2022 reached 232,081.2 tons (Badan Pusat Statistik - BPS 2024) and become one of the world's main producers of wet seaweed (Soetjipto et al., 2019). The two most common seaweeds species used are the *Euchema* spp. and *Gracilaria* spp. So far, many seaweed species have not been explored yet (Kasanah et al., 2022). Hidayah et al. (2023, 2024) evaluated secondary metabolite and methane production of eight species of tropical seaweeds from coastal areas in Gunungkidul District, Yogyakarta Province, Indonesia. The result shows that three species of tropical seaweed (*Chaetomorpha linum* (O.F.Mull.) Kutz, *Enteromorpha compressa,* and *Palisada perforate* (Bory) K.W.Nam) have potential as additives to reduce ruminant methane production due to their secondary metabolites. However, those results were only based on seaweed as a single substance, not as a supplement in ration. Napier grass is forage commonly given by farmers to ruminants as a feed. Min et al. (2022) reported that feeding grass will increase ruminant methane emission. Therefore, further study is needed to evaluate the effect of supplementing three species of tropical seaweed to Napier grass-based diets on methane gas production and rumen fermentation parameters.

Material and Methods

Treatments

There were ten treatments in this study. Napier grass (GU) was used as a basal diet (control). As treatments, three species of seaweeds were added to the basal diet in three different levels (2.50, 5.00, and 7.50% organic matter/OM), thus there were ten combination treatments including the control (GU without seaweed supplementation). As follow:

GU: Napier grass (GU)
CL2.50: GU + 2.50% OM *Chaetomorpha linum* (O.F.Mull.) Kutz
CL5.00: GU + 5.00% OM *Chaetomorpha linum* (O.F.Mull.) Kutz
CL7.50: GU + 7.50% OM *Chaetomorpha linum* (O.F.Mull.) Kutz
EC2.50: GU + 2.50% OM *Enteromorpha compressa*
EC5.00: GU + 5.00% OM *Enteromorpha compressa*
EC7.50: GU + 7.50% OM *Enteromorpha compressa*
PF2.50: GU + 2.50% OM *Palisada perforate* (Bory) K.W.Nam
PF5.00: GU + 5.00% OM *Palisada perforate* (Bory) K.W.Nam
PF7.50: GU + 7.50% OM *Palisada perforate* (Bory) K.W.Nam

Preparation of seaweeds and Napier grass

The three natural seaweeds (*Chaetomorpha linum* (O.F.Mull.) Kutz*, Enteromorpha compressa,* and *Palisada perforate* (Bory) K.W.Nam) collected from Drini and Sepanjang Beach, Gunungkidul District, Yogyakarta Province, Indonesia. Seaweeds were cleaned with water to remove sand and other debris and then dried in a freeze dryer (Buchi, Lyovapor, L-200). Napier grass used *Pennisetum purpureum* cv. Gama Umami, which was gathered from the experimental field of the Faculty of Animal Sciences, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia. The grass was dried in a 55 °C oven for 48 hours. Dried seaweeds and grasses were ground using a knife mill with a 2 mm screen.

In vitro evaluation

All samples (GU without or with supplemented seaweed at 2.50, 5.00, and 7.50% OM) were incubated *in vitro* using the Menke and Steingass (1988) method. A total of 200 mg DM sample/substrate was put into a 100 mL glass syringe (Haberle Labortechnik, Lonsee, Germany), 30 mL of fluid and rumen buffer solution was added with a ratio of 2:1 at 39 °C for 72 hours incubation. Rumen fluid was taken from the rumen of two rumen fistulated Bali cattle (male: 350 kg and female: 290 kg, body weights) aged ± 5 years at the Faculty of Animal Sciences UGM Yogyakarta, before morning feeding. Rumen fluid was filtered using four layers of cheesecloth and mixed with a buffer solution. All experimental procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine UGM Yogyakarta (Approval number: 052/EC-FKH/Eks./2022). Fistulated cattle were fed diets based on GU and wheat pollard (ratio 60:40% DM) given twice daily (08.00 and 16.00 WIB). Clean drinking water was provided at all times.

In vitro was conducted in 4 runs, each running time consisted of 10 treatments carried out in duplicate, 2 blanks, and 2 standards, with a total were 96 samples. To minimize variation between runs due to differences in rumen fluid intake, running time was set to a block. Gas productions were measured at 2, 4, 8, 12, 24, 48, and 72 hours. Calculation of gas production using the Neway program (Chen, 1994) based on the equation $Y = a + b (1 - e^{-ct})$, where Y = total volume of gas produced in time t, a = gas production from easily degraded fractions, b = gas production from potentially degraded fractions, c = gas production rate of fraction b, and total degraded and fermentable fractions (fractions a + b).

Methane production samples were measured by gas chromatography (GC 14B, Shimadzu Crop., Kyoto, Japan with Paropak column (50.0 m × 0.20 mm × 0.30 µm) and FID detector) according to Fievez, Babayemi and Demeyer (2005) method from gas samples at 24 and 48 hours of incubation. Rumen fluid samples for fermentation parameters (pH, SCFA, NH_3, rumen microbial protein) and protozoa population were collected after 72 hours of incubation. All rumen fluid samples (except pH and protozoa samples) were centrifuged for 15 minutes at 3000 rpm, transferred into 1.50 mL microtube, and then stored in a freezer for further analysis.

The pH of the supernatants was measured using a pH meter (Hanna pH-meter portable, Hanna Instruments, USA). Short chain fatty acid was determined using gas chromatography (GC 2010 Plus, Shimadzu Crop., Kyoto, Japan, HP-FFAP column (50.0 m × 0.20 mm × 0.30 µm) and FID detector) according to Cottyn and Boucque (1968) method. Ammonia determination according to the method described by Chaney and Marbach (1962) with a UV-vis microplate spectrophotometer at a wavelength of 750 nm and a standard curve ($(NH_4)_2 SO_4$, Merck) ($y = 0.028018x - 0.01108$, $r^2 = 0.99$). Microbial protein measurements were conducted according to the method described by Plummer (1967) using a UV-vis microplate spectrophotometer at a wavelength of 630 nm and standard curve (BSA/Bovine Serum Albumin, Sigma) ($y = 2.5075x + 0.0824$, $r^2 = 0.99$). The Protozoa count was determined using the Diaz, Avendro and Escobar (1997) method.

Statistical analysis

The data were analyzed in ten treatments and four replications arrangement in a randomized complete block design, with the time of rumen fluid collection as a block, using IBM SPSS Statistics version 26. All data were analyzed using analysis of variance and continued with Duncan's multiple range test to determine differences among treatments. Differences among means at $P < 0.05$ were accepted as representing statistically significant differences.

Results and Discussion

Methane gas production and protozoa population

The supplementation of *C. linum* (O.F.Mull) Kutz 2.50-5.00% OM, *E. compressa* 7.50% OM, and *P. perforate* (Bory) K.W.Nam 2.50-7.50% OM reduced ($P<0.01$) methane production at 24 and 48 hour incubation compared with control. Meanwhile, the supplementation of *C. linum* (O.F.Mull) Kutz 7.50% OM and *E. compressa* 2.50-5.00% OM increased ($P<0.01$) methane production. Table 1 showed that supplementation of 5.00% OM *P. perforate* resulted in the lowest methane production at 24 and 48 hours of incubation ($P<0.01$). Further calculations showed a decrease of methane by 83.8% at 24 hours of incubation and by 74.4% at 48 hours, which was the highest methane reduction among treatments. In concurrent with those data, the protozoa population at this treatment also showed as the lowest one (3.83×10^4 cells/mL, Table 1).

The highest reduction in methane production due to 5.00% OM *P. perforate* supplementation might be caused by secondary metabolites (phenols, tannins, flavonoids, and saponins) and nutrient (complex carbohydrates) contents of *P. perforate* (Bory) K.W.Nam. Hidayah et al. (2024) reported that *P. perforate* (Bory) K.W.Nam contains bioactive phenol, tannin, and flavonoid compounds at 0.80, 0.76, and 4.26 mg/g dry matter/DM, respectively. These bioactive compounds decreased the protozoa population (Table 1) which follows a decline of methanogenic archaea as well as an increasing rumen bacteria population (Table 3).

Tannins contribute to the decline in protozoa, which are symbiosis with methanogenic archaea, so decrease methane production. Methanogenic archaea, or protozoa population in the rumen that is either free or attached to them, can be decreased by tannin and phlorotannin (Piñeiro-Vázquez et al., 2015). Majewska et al. (2023) reported that adding tannin to Polish Lowland ewes basal diet (meadow hay (60%), barley meal (30%), soybean meal (10%) and vitamin-mineral premix) significantly reduced the number of total protozoa and *Entodinium* spp. Jayanegara et al. (2018) reported that tannins has direct effect on reducing methane by inhibiting methanogenic archaea. Previously, it had been proven that tannin monomers such as pyrogallol, gallic acids, and tannic acids have toxic properties to methanogenic archaea (Scalbert, 1991).

Similar findings were reported by Prayitno et al. (2019), when supplementation of *Gracilaria* sp. (red seaweed) at 3.60% dry matter (DM) in basal feed (60% *Cynodon dactylon* grass and 40% concentrate) with crude protein (CP) 11.8% and total digestible nutrient (TDN) 60.0% significantly reduced methane production by 49.0%. The methanogen population and protozoa were also reduced by 84.0 and 53.0%, respectively. Mihaila et al. (2022) supplementing red seaweed (*Bonnemaisonia hamifera*) with 6.00 to 10.0% OM on *Perennial ryegrass* was proven can reduce methane production by 95.4 to 98.8%, acetate proportion by 20.3 to 21.0%, and increase propionate proportion by 33.0 to 34.6%. The responses to methane production depend on the amount and species of seaweed supplementation, as a result of differences in their bioactive compounds. Differences in species, harvest time, processing during drying, starvation, and storage influence the content of seaweed bioactive compounds (Magnusson et al., 2020; Roskam et al., 2022).

The highest increase in methane occurred when the *E. compressa* 2.50% OM (lowest level) and *C. linum* (O.F.Mull) Kutz 7.50% OM (highest level) were supplemented. This condition is due to the low level of bioactive compounds from *E. compressa,* so it has not been able to reduce methanogenic archae, protozoa populations, and modify rumen microbial activity when given in small amounts. This result is linear

with the protozoa population showing the highest results in this treatment. The increase in methane gradually decreased when *E. compressa* was supplemented at a level of 5.00% OM and significantly reduced methane production with the supplementation at 7.50% OM. These results were followed by a gradual increase in total SCFA, propionate proportion, microbial protein synthesis, and a decrease in the proportion of acetate and butyrate, acetate-propionate ratio, and protozoa population (Table 1 and 3). Hidayah et al. (2023) and Hidayah et al. (2024) reported that *E. compressa* contained the lowest bioactive compounds (phenols, tannins, and flavonoids) among the seaweeds used in this study, namely 0.82 mg/g DM, 0.80 mg/g DM, and 3.39 mg/g DM.

Similar results were reported by Roskam et al. (2022) who used seven brown seaweeds (*Ascophyllum nodosum, Pelvetia canaliculata, Bifurcaria bifurcata, Cystoseira tamariscifolia, Fucus vesiculosus, Himanthalia elongata*) and one green seaweed (*Ulva intestinalis*) supplemented to basal feed (50% silage (21% DM, CP 12%) and 50% concentrate (DM 86%, CP 16%)) as much as 1% DM have not been able to reduce methane production. The supplementation of *Pelvetia canaliculata* 1% DM increased methane production by 40.40% compared to control (9.94% vs 7.08%) and there was an increase in butyrate from 8.67 mM/d to 9.82 mM/d. An increase in butrirate will be followed by an increase in methane production (Jentsch et al., 2007; Nozière et al., 2010).

Meanwhile, the supplementation of *C. linum* (O.F.Mull) Kutz with the highest level is thought to be a high level of bioactive compounds that are starting to interfere with rumen microbial activity thereby reducing the fermentation rate and products. These results can be seen from the lowest gas production up to 72 hours of incubation (Table 2), gradually decreasing total SCFA, proportion of propionate, microbial protein synthesis, and increasing the proportion of acetate and butyrate as well as the acetate-propionate ratio (Table 3) compared to the addition of *C. linum* (O.F.Mull) Kutz 2.50% OM and 5.00% OM. Hidayah et al. (2023) and Hidayah et al. (2024) reported that *C. linum* (O.F.Mull) Kutz contains the highest bioactive compounds (phenols, tannins, and flavonoids) among the seaweeds used in this study, 0.90 mg/g DM, 0.88 mg/g DM, and 9.02 mg/g DM, respectively.

Gas production

The supplementation of *C. linum* (O.F.Mull.) Kutz 2.50-5.00% OM increased (P<0.01) gas production up to 72 hours of incubation, but decreased (P<0.01) when the supplementation up to 7.50% OM. The supplementation of *E. compressa* 2.5% OM and *P. perforate* (Bory) K.W.Nam 2.50-7.50% OM increased (P<0.01) gas production up to 12 hours of incubation compared to the control. Supplementation of *P. perforate* (Bory) K.W.Nam up to 7.50% OM decreased (P<0.01) the potentially degraded fraction (b) ranged from 52.1 to 52.6 mL/200 mg DM (Table 2). Total degraded and a fermented fraction (a+b) also showed the lowest values (ranging from 52.5 to 53.0 mL/200 mg DM) due to *P. perforate* (Bory) K.W.Nam supplementation at 2.50 to 7.50% OM.

Table 1: Methane gas production and protozoa populations with supplemented different species and levels of tropical seaweed on Napier grass.

Treatments	Methane production				Methane reduction		Protozoa population (cell x 10^4/ mL)
	24 hours (mL)	48 hours (mL)	24 hours (mL/g DM)	48 hours (mL/g DM)	24 hours (%)	48 hours (%)	
GU	$4.14^{e} \pm 0.14$	$5.48^{ef} \pm 0.22$	$20.6^{e} \pm 0.97$	$27.2^{fg} \pm 1.52$	-	-	$5.01^{c} \pm 0.83$
CL2.50	$3.16^{c} \pm 0.08$	$4.40^{cd} \pm 0.18$	$15.9^{c} \pm 0.47$	$22.1^{cd} \pm 0.78$	$23.6^{d} \pm 2.18$	$19.7^{d} \pm 3.04$	$4.45^{abc} \pm 0.29$
CL5.00	$3.41^{cd} \pm 0.20$	$4.68^{d} \pm 0.23$	$17.1^{cd} \pm 1.03$	$23.5^{de} \pm 1.23$	$17.8^{c} \pm 2.23$	$14.5^{c} \pm 2.66$	$4.45^{abc} \pm 0.21$
CL7.50	$4.45^{gh} \pm 0.13$	$5.81^{f} \pm 0.21$	$22.5^{fg} \pm 0.53$	$30.0^{h} \pm 0.84$	$-7.5^{b} \pm 1.51$	$-6.08^{a} \pm 1.97$	$4.84^{bc} \pm 0.20$
EC2.50	$4.67^{h} \pm 0.11$	$5.63^{ef} \pm 0.15$	$23.7^{g} \pm 0.49$	$28.6^{gh} \pm 0.81$	$-12.7^{a} \pm 1.78$	$-0.03^{b} \pm 0.02$	$6.08^{d} \pm 0.50$
EC5.00	$4.35^{ef} \pm 0.12$	$5.38^{e} \pm 0.23$	$21.3^{ef} \pm 0.54$	$26.4^{f} \pm 0.87$	$-5.0^{b} \pm 1.27$	$1.77^{b} \pm 0.85$	$5.18^{c} \pm 0.76$
EC7.50	$3.49^{d} \pm 0.07$	$4.68^{d} \pm 0.09$	$17.7^{d} \pm 0.22$	$23.6^{e} \pm 0.25$	$15.7^{c} \pm 1.85$	$14.5^{c} \pm 1.81$	$5.18^{c} \pm 0.76$
PF2.50	$2.16^{b} \pm 0.43$	$3.18^{b} \pm 0.45$	$10.8^{b} \pm 1.95$	$15.9^{b} \pm 2.08$	$53.3^{e} \pm 5.63$	$45.5^{f} \pm 6.40$	$4.11^{ab} \pm 0.28$
PF5.00	$0.67^{a} \pm 0.13$	$1.40^{a} \pm 0.25$	$3.4^{a} \pm 0.73$	$7.0^{a} \pm 1.15$	$83.8^{f} \pm 3.78$	$74.4^{g} \pm 4.97$	$3.83^{a} \pm 0.55$
PF7.50	$3.45^{d} \pm 0.12$	$4.15^{c} \pm 0.18$	$17.7^{d} \pm 0.40$	$21.5^{c} \pm 1.24$	$16.6^{c} \pm 1.59$	$24.3^{e} \pm 1.56$	$4.33^{abc} \pm 0.34$

Notes: Means in the same column with different superscripts differ significantly (P<0.01); DM: dry matter; GU: Napier grass (*Pennisetum purpureum* cv. Gama Umami); CL2.50: GU + *C. linum* (O.F.Mull.) Kutz 2.50% OM; CL5.00: GU + *C. linum* (O.F.Mull.) Kutz 5.00% OM; CL7.50: GU + *C. linum* (O.F.Mull.) Kutz 7.50% OM; EC2.50: GU + *E. compressa* 2.50% OM; EC5.00: GU + *E. compressa* 5.00% OM; EC7.50: GU + *E. compressa* 7.50% OM; PF2.50: GU + *P. perforate* (Bory) K.W.Nam 2.50% OM; PF5.00: GU + *P. perforate* (Bory) K.W.Nam 5.00% OM; PF7.50: GU + *P. perforate* (Bory) K.W.Nam 7.50% OM.

Table 2: Total gas production with supplemented different species and levels of tropical seaweed on Napier grass.

Treatments	Gas production (mL/200 mg DM)										
	2 hours	4 hours	8 hours	12 hours	24 hours	48 hours	72 hours	a (mL/200 mg DM)	b (mL/200 mg DM)	c (mL/hour)	a+b (mL/200 mg DM)
GU	3.71^{b} ± 0.97	6.71^{b} ± 0.89	12.7^{bc} ± 1.05	19.3^{b} ± 1.47	36.75^{bc} ± 2.50	50.05^{c} ± 1.51	55.65^{bcd} ± 0.64	-1.14 ± 2.10	63.63^{bc} ± 2.35	0.038^{ab} ± 0.00	62.48^{cd} ± 4.16
CL2.50	9.05^{e} ± 1.20	12.05^{d} ± 1.73	18.6^{f} ± 2.16	25.7^{f} ± 2.78	43.18^{d} ± 3.31	55.67^{de} ± 4.38	61.67^{ef} ± 4.23	-2.34 ± 2.50	61.17^{bc} ± 4.10	0.039^{abc} ± 0.00	58.82^{bc} ± 6.60
CL5.00	7.12^{d} ± 1.11	10.24^{cd} ± 1.30	17.2^{ef} ± 1.21	24.7^{def} ± 1.57	43.07^{d} ± 2.16	56.68^{e} ± 2.21	62.42^{f} ± 2.53	0.36 ± 1.42	65.33^{c} ± 2.77	0.040^{abc} ± 0.00	65.69^{d} ± 1.67
CL7.50	0.58^{a} ± 0.24	3.16^{a} ± 0.51	8.09^{a} ± 2.16	14.9^{a} ± 0.76	31.34^{a} ± 0.60	43.68^{a} ± 0.80	48.84^{a} ± 1.29	-0.84 ± 4.05	60.55^{b} ± 3.44	0.038^{ab} ± 0.00	59.70^{cd} ± 6.90
EC2.50	5.35^{c} ± 0.65	9.30^{c} ± 0.59	16.0^{de} ± 1.81	24.2^{def} ± 2.01	39.38^{c} ± 3.19	52.10^{cd} ± 4.00	58.20^{de} ± 4.18	0.58 ± 1.81	62.46^{bc} ± 2.46	0.040 ± 0.00	63.04^{cd} ± 3.62
EC5.00	3.59^{b} ± 1.10	6.69^{b} ± 0.95	12.6^{bc} ± 0.81	19.8^{bc} ± 1.03	36.90^{bc} ± 2.28	48.78^{bc} ± 2.68	54.85^{bcd} ± 2.67	-2.19 ± 0.51	61.43^{bc} ± 2.59	0.039^{ab} ± 0.00	59.24^{bc} ± 3.09
EC7.50	3.66^{b} ± 0.26	6.45^{b} ± 0.82	11.5^{b} ± 1.73	18.0^{b} ± 1.19	34.32^{ab} ± 1.01	45.19^{ab} ± 1.29	51.96^{ab} ± 0.07	-2.38 ± 0.76	59.57^{b} ± 2.22	0.036^{a} ± 0.00	57.19^{abc} ± 2.31
PF2.50	8.55^{e} ± 1.39	11.26^{d} ± 1.25	17.4^{ef} ± 0.88	25.1^{ef} ± 1.66	40.21^{cd} ± 1.93	51.55^{c} ± 1.81	56.30^{cd} ± 0.62	0.35 ± 2.45	52.11^{a} ± 3.18	0.042^{bc} ± 0.00	52.46^{a} ± 2.29
PF5.00	6.62^{cd} ± 1.34	9.20^{c} ± 2.12	15.5^{de} ± 1.14	22.6^{de} ± 2.35	37.79^{bc} ± 2.24	48.04^{bc} ± 1.95	52.71^{abc} ± 2.86	0.29 ± 1.13	52.64^{a} ± 1.46	0.042^{bc} ± 0.00	52.93^{ab} ± 1.06
PF7.50	5.52^{c} ± 0.45	8.24^{bc} ± 1.37	14.7^{cd} ± 1.71	22.2^{cd} ± 1.82	36.99^{bc} ± 2.06	48.06^{bc} ± 1.93	52.82^{abc} ± 1.96	0.54 ± 1.74	52.47^{a} ± 2.15	0.044^{c} ± 0.00	53.01^{ab} ± 2.99

Notes: Means in the same column with different superscripts differ significantly (P<0.01); DM: dry matter; GU: Napier grass (*Pennisetum purpureum* cv. Gama Umami); CL2.50: GU + *C. linum* (O.F.Mull.) Kutz 2.50% OM; CL5.00: GU + *C. linum* (O.F.Mull.) Kutz 5.00% OM; CL7.50: GU + *C. linum* (O.F.Mull.) Kutz 7.50% OM; EC2.50: GU + *E. compressa* 2.50% OM; EC5.00: GU + *E. compressa* 5.00% OM; EC7.50: GU + *E. compressa* 7.50% OM; PF2.50: GU + *P. perforate* (Bory) K.W.Nam 2.50% OM; PF5.00: GU + *P. perforate* (Bory) K.W.Nam 5.00% OM; PF7.50: GU + *P. perforate* (Bory) K.W.Nam 7.50% OM.

The increase in gas production with the seaweed supplementation on GU is thought to be a small amount of additional minerals from seaweed which can stimulate rumen microbial activity thereby increasing the fermentation rate. Pino and Heinrichs (2016) explained that higher amounts of minerals can help ruminants stimulate rumen microbes and fermentation. However, if the mineral content is too high, it can reduce feed degradation so that gas production decreases. This can be shown from the treatment with the supplementation of *C. linum* (O.F.Mull) Kutz 7.50% OM which contains the highest minerals (14.99% DM) among all treatments which have the lowest gas production. Minerals will interact with seaweed fibers (some polysaccharides) such as alginate and agar or carrageenan which form insoluble complexes (Circuncisão et al., 2018). The same results were reported by Ahmed et al. (2022), there was an increase in gas production with the supplementation of seven species of brown seaweed (*Ascophyllum nodosum, Ecklonia maxima, Sargassum fulvellum, Lessonia flavicans, Lessonia nigrescens, Laminaria japonica*) as much as 20% DM in basal feed (50% hay and 50% concentrate).

Meanwhile, the low values of fraction b and fraction a+b at *P. perforate* (Bory) K.W.Nam supplementation at 2.50 to 7.50% OM treatments were related to the tannin compound of seaweed that protects protein from rumen microbial degradation. This was indicated by the protein degradation results (ammonia concentration; Table 3) that were similar to the control treatment, although their protein content was different (10.9 vs. 11.2% DM). *P. perforate* (Bory) K.W.Nam contains tannins of 0.76 mg/g DM (Hidayah et al., 2024). Rodríguez et al. (2014) stated that tannins are natural protectors of protein in ruminant livestock. Tannins form stable complexes with proteins at normal rumen pH but dissociate in the abomasum (Getachew, Makkar, & Becker, 2000). Apart from that, tannins also bind fiber and carbohydrates, thereby reducing its degradation in the rumen so that gas production decreases. Mueller (2006) explained that tannins form complexes with natural polymers such as proteins and carbohydrates. Polyphenols (such as tannin) have been reported to slow down the breakdown of protein and fiber in the rumen, by preventing microbes from attaching them (Makkar, 2003). The same results were reported by Machado et al. (2016), in which a 25.0% reduction in total gas production due to 2.00% OM *Asparagopsis taxiformis* supplementation in Rhodes grass (*Chloris gayana)* hay was noted. The inclusion of seaweeds in the concentrate of a diet up to 200 g/kg has decreased average gas production rate to control (de la Moneda et al., 2019).

Rumen fermentation parameters

The rumen pH value in all treatments at normal conditions, namely in the range of 6.86-6.93. The supplementation of all seaweed species increase (P<0.01) total SCFA production, proportion of propionate, microbial protein synthesis, and reduce the proportion of acetate, butyrate, and acetate-propionate ratio compared to the control. The supplementation of *P. perforate* (Bory) K.W.Nam 5.00% OM resulted in the highest total VFA production,

proportion of propionate, microbial protein synthesis, and the lowest proportion of acetate, butyrate, and acetate propionate ratio. The next best results were when GU supplemented *E. compressa* 7.5% OM. Ammonia (NH_3) production was the same as control (P>0.01) with the supplementation of all seaweed species with 7.50% OM. This condition indicates that the supplementation of seaweed used in the study up to 7.50% OM did not increase protein degradation in the rumen. The addition of *C. linum* (O.F.Mull.) Kutz and *P. perforate* (Bory) K.W.Nam 2.50-7.50% increase (P<0.01) microbial protein synthesis. The results of fermentation parameters are presented in Table 3.

The pH value is still within the normal range, indicating that the supplementation of seaweed does not disturb fermentation activity. Zheng et al. (2020) stated that normal pH values can vary around 5.5-7.5 depending on the type of feed and frequency of feeding. A pH value below 6.2 has a negative effect on fiber fraction digestibility, while a pH value below 5.5 will impact the incidence of rumen acidosis, reduce consumption, and even in extreme conditions the livestock will not want to eat (off feed) and can cause serious problems (Franzolin & Dehority, 2010).

The NH_3 concentration in this study ranged from 13.12 to 14.99 mg/100 mL, which is sufficient to support microbial protein synthesis. Satter and Slyter (1974) stated that the NH_3 concentrations for seaweed were above the level limiting *in vitro* ruminal microbial growth (5 mg/100 mL). The supplementation of *P. perforate* (Bory) K.W.Nam 2.50-7.50% OM produced the highest microbial protein synthesis which to be due to the mineral content of *P. perforate* (Bory) K.W.Nam (sulfur and phosphorus) being the highest among the seaweeds used, namely in 0.81% DM and 0.09% DM (Hidayah et al., 2023). Pathak (2008) explained that minerals and vitamins are one of the factors that can increase microbial protein synthesis, especially the minerals sulfur and phosphorus which are very important in microbial growth. Sulfur is needed in the process of methionine and cysteine synthesis (National Research Council - NRC, 2001) and phosphorus is needed for ATP and protein synthesis by rumen microbes (Pathak, 2008). Pathak (2008) also explained that a lack of phosphorus stock can limit microbial growth.

The nutrient content and bioactive compounds of *P. perforate* (Bory) K.W.Nam, which is red seaweed, are better to stimulate to increase SCFA and shift the metabolic pathway from methanogenesis, resulting increase the proportion of propionate and a decrease in acetate and butyrate, followed by low methane production. This result is linear with a decrease in methane production which tends to be higher when using red seaweed compared to green seaweed (*C. linum* (O.F.Mull) Kutz and *E. compressa*) that used in this study. De Bhowmick and Hayes (2023) stated that *in vitro*, the use of red seaweed in ruminants was more capable of reducing methane, namely by 60-90% while green seaweed was only 45%. The same research results were reported by Machado et al. (2016) which supplemented to *A.s taxiformis* (red seaweed) flour to Rhodes grass hay (C. gayana) with 2% OM can increase propionate proportion (19.2% vs 28.7%) and reduce acetate proportion (75% vs 60.4%) and acetate-propionate ratio (3.9 vs 2.1), as well as 95% methane gas. Mihaila et al. (2022) also reported that the use of red seaweed *B. hamifera* supplemented to Perennial ryegrass as a basal feed at 6-10% OM can increase 32.98-34.58% propionate proportion and reduce 20.27-21.02% acetate proportion, and 95.4-98.8% methane production.

Table 3: Rumen fermentation parameters with supplemented different species and levels of tropical seaweed on Napier grass.

Treatments	pH	Total SCFA (mM)	Acetate (%)	Propionate (%)	Butyrate (%)	Acetate Propionate ratio	NH_3 (mg/100 mL)	Rumen microbial protein (mg/100 mL)
GU	$6.88^{ab} \pm 0.05$	$32.8^{a} \pm 2.70$	$79.0^{e} \pm 0.68$	$5.24^{a} \pm 1.04$	$15.8^{g} \pm 0.83$	$15.6^{d} \pm 3.36$	$13.1^{a} \pm 0.20$	$63.1^{a} \pm 0.25$
CL2.50	$6.86^{a} \pm 0.07$	$54.0^{b} \pm 4.94$	$75.3^{bc} \pm 0.69$	$12.5^{c} \pm 1.78$	$12.3^{ef} \pm 1.24$	$6.12^{bc} \pm 0.82$	$15.0^{c} \pm 0.57$	$75.9^{c} \pm 1.23$
CL5.00	$6.90^{ab} \pm 0.01$	$53.4^{b} \pm 7.55$	$75.8^{cd} \pm 0.38$	$12.1^{bc} \pm 0.55$	$12.1^{e} \pm 0.28$	$6.26^{bc} \pm 0.32$	$14.7^{bc} \pm 0.92$	$74.9^{c} \pm 1.19$
CL7.50	$6.93^{b} \pm 0.05$	$45.4^{b} \pm 2.34$	$76.3^{cd} \pm 0.40$	$10.5^{b} \pm 1.09$	$13.2^{f} \pm 1.05$	$7.30^{c} \pm 0.72$	$14.1^{abc} \pm 0.57$	$74.0^{c} \pm 1.10$
EC2.50	$6.91^{ab} \pm 0.02$	$56.4^{b} \pm 5.04$	$75.0^{def} \pm 0.39$	$13.4^{cd} \pm 1.46$	$11.6^{de} \pm 1.09$	$5.68^{abc} \pm 0.71$	$14.9^{c} \pm 0.91$	$62.0^{a} \pm 1.08$
EC5.00	$6.89^{ab} \pm 0.02$	$69.2^{c} \pm 13.38$	$73.9^{ab} \pm 1.22$	$15.4^{de} \pm 2.11$	$10.9^{cd} \pm 0.91$	$4.96^{ab} \pm 0.76$	$14.9^{c} \pm 0.85$	$65.6^{ab} \pm 1.80$
EC7.50	$6.86^{a} \pm 0.01$	$90.3^{d} \pm 1.89$	$73.2^{a} \pm 0.34$	$16.7^{e} \pm 0.25$	$10.1^{bc} \pm 0.52$	$4.38^{ab} \pm 0.05$	$14.1^{abc} \pm 0.35$	$69.0^{b} \pm 2.05$
PF2.50	$6.92^{ab} \pm 0.01$	$51.0^{b} \pm 6.68$	$73.3^{a} \pm 2.06$	$16.1^{e} \pm 1.98$	$10.6^{cd} \pm 0.61$	$4.63^{ab} \pm 0.68$	$13.7^{ab} \pm 0.98$	$118.0^{d} \pm 5.75$
PF5.00	$6.90^{ab} \pm 0.04$	$111.8^{e} \pm 10.18$	$75.8^{bc} \pm 0.95$	$19.0^{f} \pm 1.00$	$5.9^{a} \pm 0.22$	$3.97^{a} \pm 0.26$	$14.4^{bc} \pm 0.76$	$119.1^{d} \pm 5.29$
PF7.50	$6.91^{ab} \pm 0.04$	$45.1^{b} \pm 4.53$	$76.9^{d} \pm 1.03$	$14.0^{cd} \pm 0.64$	$9.13^{b} \pm 0.44$	$5.51^{abc} \pm 0.32$	$13.9^{abc} \pm 1.11$	$117.5^{d} \pm 4.85$

Notes: Means in the same column with different superscripts differ significantly (P<0.01); DM: dry matter; GU: Napier grass (*Pennisetum purpureum* cv. Gama Umami); CL2.50: GU + *C. linum* (O.F.Mull.) Kutz 2.50% OM; CL5.00: GU + *C. linum* (O.F.Mull.) Kutz 5.00% OM; CL7.50: GU + *C. linum* (O.F.Mull.) Kutz 7.50% OM; EC2.50: GU + *E. compressa* 2.50% OM; EC5.00: GU + *E. compressa* 5.00% OM; EC7.50: GU + *E. compressa* 7.50% OM; PF2.50: GU + *P. perforate* (Bory) K.W.Nam 2.50% OM; PF5.00: GU + *P. perforate* (Bory) K.W.Nam 5.00% OM; PF7.50: GU + *P. perforate* (Bory) K.W.Nam 7.50% OM.

Conclusions

Supplementation of *Enteromorpha compressa* 2.50% OM and *Chaetomorpha linum* (O.F.Mull) Kutz 7.50% OM on Napier grass (*Pennisetum purpureum* cv. Gama Umami) increased methane in 24 hours incubation at 12.72% and 48 hours incubation at 6.08%, respectively. Meanwhile, *Palisada perforate* at 5.00% OM supplemented decreased methane production as well as increasing total SCFA and rumen microbial protein. *P. perforate* (Bory) K.W.Nam can be used as a potential agent of methane reduction in ruminants so enhance their production.

Authors contribution

Conceptual idea: Hidayah, N.; Kustantinah; Astuti, A.; Noviandi, C.T.; Methodology design: Hidayah, N.; Kustantinah; Astuti, A.; Noviandi, C.T.; Data collection: Hidayah, N., Data analysis and interpretation: Hidayah, N., Noviandi, C.T.; and Writing and editing: Hidayah, N.; Kustantinah; Astuti, A.; Noviandi, C.T.

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