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Research article

Evaluation of rumen in vitro gas production and fermentation characteristics of four tropical seaweed species

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Abstract

Studies on the identification and characterization of numerous seaweed species from tropical oceans have not been widely reported. The objective of this study was to evaluate the rumen in vitro gas production and fermentation characteristics of four tropical seaweed species. The design of treatments was a randomized complete block design with four different seaweed species (brown seaweed: Laminaria sp. and Padina australis; red seaweed: Gracilaria sp. and Eucheuma cottonii) and four replications for each treatment. For the gas production, easily degraded fraction (a), potentially degraded fraction (b), rate of gas production of b fraction (c), and total fraction degraded (a+b) parameters of brown seaweed were higher and faster than those of red seaweed. The lowest methane production at 24 hours incubation was obtained for Gracilaria sp., which reduced methane production from blank (44.38%) and standard (Pangola substrate) samples (60.63%), followed by Padina australis at 28.98 and 49.73% respectively. Padina australis resulted in the highest propionate proportion (16.03%), lowest butyrate (11.92%) and A:P ratio (4.52) ($P<0.05$). There were no differences in NH3 (34.17-37.31 mg/100 mL) or microbe protein concentration (9.03-10.60 mg/100 mL) among the seaweed species. It was concluded that brown seaweed (Laminaria sp. and Padina australis) were more degradable than red seaweed (Gracilaria sp. and Eucheuma cottonii) in the rumen. Padina australis is the most potential as ruminant feed because it resulted in the highest propionate proportion, the lowest butyrate and A:P ratio, and low methane production and did not disturb the NH3 and microbe protein concentrations.

Keywords: Degradability, In vitro fermentation, Ruminant feed, Tropical macroalga

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INTRODUCTION

Ruminants such as cattle, goats, and sheep produce methane (CH_4) gas emissions from two sources, there are enteric fermentation and manure. Dini et al. (2012) reported that the highest of enteric methane gas production is in the rumen (87-90%) and in the large intestine (10-13%). Methane gas is formed naturally through methanogenesis as a mechanism to avoid hydrogen accumulation during feed fermentation in the rumen. Methanogenesis is carried out by methanogenic bacteria by reducing CO_2 with H_2 and forming methane (CH_4), then excreted 83% through eructation, 16% through respiration, and 1% through the anus (Yanuartono et al., 2019). Methane gas from enteric fermentation is a by-product of the ruminant digestion process and is produced by methanogenic microorganisms, namely *Archaea* during the methanogenesis process (Broucek, 2014). The effort to reduce ruminant methane emission is important to do because methane emission has two negative effects: it contributes 17% to greenhouse gas production (Pandey et al., 2021) and reduces ruminant production because approximately 3-12% of the digested energy from the feed is lost as methane as stated by Mayberry et al. (2019).

Recent research reported that seaweed or marine macroalgae has the potential for ruminant feed. Seaweed contains abundant of polysaccharide, amino acids, vitamins, natural metabolites, and minerals essential for metabolic functions (Min et al., 2021). The use of seaweed increased the feed conversion efficiency, growth rate, and productivity of ruminants (Min et al., 2021). Several studies have reported that seaweed reduce ruminants methane emissions very efficient due to the content of their metabolites, precisely halogen compounds and phlorotannin (Belanche et al., 2016; Roque et al., 2019; Kinley et al., 2020; Choi et al., 2021; Min et al., 2021) Halogen compounds such as bromoform are antimethanogenic compounds that can inhibit the methyl-coenzyme reductase (MCR) enzyme in the methanogenesis process (Allen et al., 2014; Costa and Leigh, 2014; Kinley et al., 2016). Phlorotannin can affect the integrity of the microbial cell and wall membranes, causing microbial cell lysis and disrupted growth (Hierholtzer et al., 2013). Phlorotannin can inactivate extracellular enzymes and proteins required for microorganism growth and metabolism (Scalbert, 1991). Sarwono et al. (2019) stated that bioactive compounds such as phloroglucinol (the monomer unit of phlorotannin) decreased CH_4 production by reducing the methanogen population. The reduced CH_4 and CO_2 production will lead to less energy losses with high Volatile fatty acid (VFA) production (Choi et al., 2021).

Indonesia is a tropical country with a large diversity of seaweed. Indonesian Statistic Centre (BPS, 2021) reported that Indonesia produced seaweed in 2020, reaching 8.5 million tons. Studies on the digestibility and fermentation characteristics of many seaweed species from tropical oceans have not been widely reported. This objective of this study was to evaluate on the rumen in vitro gas production and fermentation characteristics of four tropical seaweed, namely *Laminaria* sp. and *Padina australis* (brown seaweed) and *Gracilaria* sp. and *Eucheuma cottonii* (red seaweed).

MATERIALS AND METHODS

Ethical approval

The research procedure was approved by the standard of the ethical procedure from the Animal Care and Use Committee No: 052/EC-FKH/Eks./2022 Faculty of Veterinary Medicine Universitas Gadjah Mada, Indonesia.

Sample collection and preparation

The natural seaweed species used in this research were from Kalapa Beach, Tuban, East Java, Indonesia, in April 2022. There were two species of brown seaweed (*Laminaria* sp. and *Padina* australis) and two species of red seaweed (*Gracilaria* sp. and *Eucheuma cottonii*). The seaweed were cleaned with water and dried under the sun until they reached a stable weight. After that, all the seaweed were ground to a fine powder. The rumen fluid in this experiment was compiled from two rumens fistulated from Bali beef cattle before morning feeding at the Faculty of Animal Science, Universitas Gadjah Mada, Indonesia. The feed of the fistulated animals consisted of pollard and king grass (*Pennisetum purpureum*) with a ratio of 40:60% DM.

In vitro evaluation, sampling, and calculation

All seaweed samples were incubated *in vitro* gas technique method according to the Menke and Steingass (Menke and Steingass, 1988). An amount of 200 mg DM substrate (seaweed powder) was place in a 100 mL glass syringes (Haberle Labortechnik, Lonsee, Germany) and added to 30 mL rumen liquor-buffer solution (1:2) at 39°C for 72 hours. The gas production was measured at 2, 4, 8, 12, 24, 48, and 72 h. Samples for fermentation characteristics (VFA, NH_3, and rumen microbial protein) analysis were taken from the aliquot after 72 h incubation and then centrifuged for 15 minutes with 8 gravitations. Calculation of the gas production using the Neway program (Chen, 1994) based on equation $Y= a+b\ (1-e^{-ct})$, where Y= volume of total gas produced in time t, a= gas production from easily degraded fraction, b= gas production from potentially tainted fraction, c= rate of gas production of b fraction, and total fraction degraded and fermented (a+b fraction). Gas samples were measured for methane emissions according to the (Fievez et al., 2005) method. Volatile fatty acid (VFA) concentrations were determined with gas chromatography. A total of 1 mL supernatant was added to 20% (200 µL) meta-phosphoric acid in a tube and analysed using gas chromatography (GC 8A, Shimadzu Crop., Kyoto, Japan, capillary column type containing 10% SP-1200, 1% H3PO4 on 80/100 Cromosorb WAW and nitrogen as a gas carrier). For ammonia and microbial protein measurements, 1 mL supernatant was centrifuged at 3,000 and 10,000 rpm for 15 minutes and then analysed with a spectrophotometer. Analysis of ammonia based on the indophenol reaction (reaction between ammonia and sodium phenate) as explained by Weatherburn (1967) and microbial protein was determined by the (Plummer, 1967) method.

Study design and data analysis

The design of the experiment was a randomized complete block design with four types of seaweed species (*Laminaria* sp., *Padina* australis, *Gracilaria* sp. and *Eucheuma cottonii*) with four replications in each treatment. The time of rumen fluid collection as a block design. Data were analysed statistically using analysis of variance (ANOVA), and the differences among treatment means were examined by Duncan's multiple range test (Steel and Torrie, 1995). Statistical analysis was conducted using IBM SPSS Statistics version 26.

RESULTS

In vitro total gas and methane production

The total gas production was significantly different (P<0.01) among the treatments at all observed incubation times. *Laminaria* sp. has the highest gas production from 2 until 72 hours incubation, following by *Padina australis* at 8 until 48 hours incubation in the second highest gas production. *Gracilaria* sp. has the lowest gas production and following by *Eucheuma cottonii* from 2 until 72 hours incubation. For the gas production curve, the easily degraded fraction (a), potentially degraded fraction (b), and the rate of gas production of b fraction (c), and total fraction degraded (a+b) values for the of brown seaweed were higher and faster than those of red seaweed, with the highest obtained for *Laminaria sp*. and followed by *Padina australis*. The highest methane production at 24 hours incubation was obtained for *Laminaria* sp. and *Eucheuma cottonii* substrates, while the lowest for *Gracilaria* sp. (Tables 1 and 2). *Gracilaria* sp. can reduce methane production from blank at 44.38% and standard substrate (Pangola substrate) samples at 60.63%. Meanwhile, *Padina australis* can reduce methane production from blank and standard at 28.98 and 49.73% respectively.

Table 1 Cumulative gas production (ml/200 mg DM) of four tropical seaweed species over 72 hours

Seaweed species	2	4	8	12	24	48	72
Brown seaweed							
Laminaria sp.	8.09^{b}	11.17^{b}	15.78^{b}	19.50^{b}	28.47^{b}	37.32^{c}	38.87^{b}
Padina australis	5.53^{a}	9.22^{a}	13.44^{ab}	16.87^{ab}	25.17^{ab}	33.07^{b}	33.19^{a}
Red seaweed							
Gracilaria sp.	4.72^{a}	7.64^{a}	11.85^{a}	15.03^{a}	21.78^{a}	28.40^{a}	28. 53^{a}
Eucheuma cottoni	3.82^{a}	7.09^{a}	11.87^{a}	15.28^{a}	22.79^{a}	30.02^{ab}	31.94^{a}
SEM	2.10	2.15	2.40	2.76	3.88	4.64	4.95

$^{a-c}$Means in the same columns with different superscripts differ very significantly (P<0.01)

Table 2 Gas production over 72 hours of incubation (a, b, c, and a+b fractions) and methane emissions of four tropical seaweed species

Variables	Brown seaweed		Red seaweed		SEM
	Laminaria sp.	*Padina australis*	*Gracilaria* sp.	*Eucheuma cottoni*	
a (mL/200 mg DM)	5.07[b]	2.66[a]	2.02[a]	1.21[a]	1.99
b (mL/200 mg DM)	35.89[c]	32.12[b]	27.60[a]	31.75[b]	4.08
c (mL/hours)	0.044[a]	0.050[b]	0.054[b]	0.049[ab]	0.01
a+b (mL/200 mg DM)	40.96[c]	34.78[b]	29.62[a]	32.97[ab]	5.92
$CH_{4.24}$ (mL/g DM)	18.41[c]	13.00[ab]	9.81[a]	14.63[bc]	4.18

[a-c]Means in the same columns with different superscripts differ very significantly (P<0.01)

In vitro rumen fermentation

The total VFA and acetate proportion did not differ significantly among all seaweed species (P>0.05). *Padina australis* resulted in the highest propionate proportion (15.66%) and the lowest butyrate (11.92%) and A:P ratio (4.52). Meanwhile, *Eucheuma cottoni* resulted in the lowest total propionate proportion (6.72%) and the highest butyrate (15.22%) and Acetate: Propionate (A:P) ratio (P<0.05). There were no differences in NH_3 (34.17-37.31 mg/100 mL) or microbial protein concentration (9.03-10.60 mg/100 mL) among all seaweed species (P<0.05) (Table 3).

Table 3 Fermentation characteristics of four tropical seaweed species over 72 hours

Variables	Brown seaweed		Red seaweed		SEM
	Laminaria sp.	*Padina australis*	*Gracilaria* sp.	*Eucheuma cottoni*	
Total VFA (mM)	57.05	78.21	59.99	37.52	18.61
Acetate (%)	71.06	74.23	73.87	76.57	3.45
Propionate (%)	11.08[b]	16.03[c]	12.62[b]	6.25[a]	3.70
Butyrate (%)	15.95[b]	11.92[a]	14.31[ab]	15.22[b]	2.58
A/P	6.63[a]	4.52[a]	5.86[a]	12.94[b]	3.49
NH_3 (mg/100 mL)	36.77	37.31	36.05	34.17	2.58
Rumen microbial protein (mg/100 mL)	9.63	9.03	9.91	9.85	1.41

[a-c]Means within the same row with different superscripts differ significantly (p < 0.01)

DISCUSSION

Based on our previous study that reported by Kustantinah et al. (2022), the nutrient analysis four species seaweed in this research included dry matter (DM), organic matter (OM), ash, crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen free extract (NFE), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose, and gross energy respectively are *Laminaria* sp. (24.54%; 43.27%; 56.73%; 12.28%; 0.93%; 4.98%; 25.08%; 29.48%; 8.13%; 21.35%; 1,575 Cal/g), *Padina australis* (30.59%; 58.56%; 41.44%; 12.57%; 0.37%; 6.31%; 39.31%; 22.75%; 6.90%; 15.85%; 1,896 Cal/g), *Gracilaria* sp. (22.58%; 34.37%; 65.63%; 11.72%; 0.21%; 2.49%;

19.95%; 21.74%; 5.46%; 16.28%; 1,083 Cal/g), and *Eucheuma cottonii* (13.67%; 76.58%; 23.42%; 8.55%; 2.85%; 8.80%; 56.38%; 56.93%; 11.44%; 45.49%; 2,911 Cal/g).

The cumulative and kinetic gas production of red seaweed lower than brown seaweed presumably due to red seaweed containing a low NFE (*Gracilaria* sp. at 19.95%) and high NDF-ADF (*Eucheuma cottonii* at 56.93%-11.44%), whereas brown seaweed (*Laminaria* sp. and *Padina australis*) high NFE (25.08% and 39.31%) and low NDF-ADF (22.75-6.90% and 29.48-8.13%). The conditions indicated that the high carbohydrate and carbohydrate structure in brown seaweed were more easily degraded by rumen microbes, hence increasing gas production. Getachew et al. (2004) stated that there was a strong positive correlation between non fibre carbohydrate and gas production. Jung et al. (2011) reported that the carbohydrate content of *Laminaria japonica* (51.90–59.70%) was higher than that of *Gracilaria verrucosa* (33.50%). *Laminaria* sp. is a brown seaweed, and the main carbohydrates are laminarin, alginates, and mannitol, while *Gracilaria* sp. are red seaweed which are mainly composed of agarose, agaropectin, cellulose, and carrageenan (Park et al., 2012). Potential gas production had a negative correlation with ADF content ($r = -0.60$; $p < 0.05$); the potential gas production decreased with increasing NDF and ADF content (Kulivand and Kafilzadeh, 2015).

The gas production up to 72 hours of incubation in this research resulted at 28.50 - 36.63 ml/200 mg DM. This gas production was not higher than 60 mL/200 mg DM, which indicated that the organic matter degradation of all seaweed species in this study were relatively low (Adiwimarta et al., 2017). This was possibly due to low organic matter (34.37-76.58%) and high minerals (23.42-65.65%) of the seaweed used in this study. Matanjun et al. (2009) reported similar results when using three tropical edible seaweeds, *Caulerpa lentillifera* (Chlorophyta/green seaweed), *Sargassum polycystum* (Phaeophyta/brown seaweed), and *Eucheuma cottonii* (Rhodophyta/red seaweed), which were high in ash (37.15–46.19%). The values of a (1.12-5.97 mL/200 mg DM) and b (26.47-43.38 mL/200 mg DM) indicated that seaweed in this study had a lower easily degraded fraction than the potentially degraded fraction. On the contrary, results reported by Dubois et al. (2013); showed that the tropical marine macroalga (red and brown) from intertidal reefs around Townsville, QLD, Australia had a higher easily degraded fraction (35.50-83.85 gas mL) than the potentially degraded fraction (2.46-4.17 gas mL). This was probably due to the tropical marine macroalga (red and brown) from intertidal reefs around Townsville, QLD, Australia having higher organic matter (43.60-90.02% DM) than the tropical seaweedin this study (34.37-76.58% DM), so they were more degradable in the rumen.

Gas production is an indication of quantitative VFA production (Getachew et al., 2004). However, in this study, *Laminaria* sp. have the highest gas production, but results showed that the highest VFA obtained for *Padina australis* (Table 3). This suggests that *Padina australis* may have a higher tannin and phlorotannin contents than *Laminaria* sp. ss proposed by Gupta and Abu-Ghannam (2011). These authors stated that brown seaweed produces phlorotannins and a range of other natural products such as phlorotannins. Phlorotannins are similar to tannins of terrestrial plants; phenolic compounds contain a great number of hydroxyl groups, which are highly soluble in water, bind strongly to proteins, polysaccharides, and other biopolymers, chelate divalent metals, and have a polymeric structure (Imbs and Zvyagintseva, 2018). Min et al. (2021) reported that dietary tannins can decrease the utilization of nutrients by directly inhibiting microbial activity or indirectly by forming complexes with the nutrients. Tannin can reduce the process of degradation in the rumen due to bonding organic matter, which in turn lowers gas production (Adiwimarta et al., 2017).

Laminaria sp. had the highest methane production at 24 hours of incubation which was linear with the highest gas production. This indicates that *Laminaria* sp. are more degradable in the rumen, but the secondary metabolites (tannin and phlorotannin) at low levels are not able to decrease methane production. Machado et al. (2014) stated that the reduction in total gas production and methane production is associated with secondary metabolites. In this study *Gracilaria* sp. had the lowest methane production, which was probably due to the low CF, NDF, and ADF contents (Kulivand and Kafilzadeh, 2015) reported that NDF and ADF have a positive correlation with methane production (r = 0.75 and 0.77, $p<0.01$). Higher NDF reises methane production by moving the short-chain fatty acid proportion towards acetate, which produces more hydrogen (Carulla et al., 2005; Puchala et al., 2005; Tavendale et al., 2005). The high soluble carbohydrate content is offered to facilitate the production of propionate in the rumen, lower ruminal pH and infere methanogen growth, thereby reducing methane production per unit of fermented OM (Van Kessel and Russell, 1996; Meale et al., 2012). As reported by Molina-Alcaide et al. (2017), who used seaweed from the northern Norway ocean, *Palmaria palmata* which had the high NDF, and low nonstructural carbohydrates exhibited the highest asymptotic gas and methane production. The opposite was true for *Alaria esculenta*, which had the lowest asymptotic gas and methane production and the lowest NDF and highest NDF contents (Molina-Alcaide et al., 2017)

Padina australis had the highest of fermentation characteristic than others because it containing high NFE (39.31%) and low NDF-ADF (22.75%-6.90%). Getachew et al. (2004) stated that feed with a high non fiber carbohydrate (NFC) content proportionally increased the propionate content and reduced the acetate to propionate ratio. The NDF structure is more complex than starch and takes longer time to ferment, resulting in low VFA and high acetate and butyrate production (Penner et al., 2009; Martínez et al., 2010). *Padina australis* also contained more polysaccharides than the others. Wang et al. (2018) stated that propionate is one of the final products from plant polysaccharides fermented by rumen microbes. *Eucheuma cottoni* contained the highest crude fiber, NDF, and ADF contents at 8.80%, 56.93%, and 11.44% DM respectively. These authors showed that a diet with high forage (70%) produced high acetate and that a diet with high concentrate (35% forage) produced high propionate. Blaxter (1962) stated that starch-decomposing bacteria produced propionate and fiber-degrading bacteria produced acetate.

The CP content of all seaweed in this study was almost similar (less than 15%) and did not have an effect on reducing ammonia and increasing rumen microbial protein. Likewise, the content of seaweed bioactive compounds also did not have an effect. Research by (de la Moneda et al., 2019) reported that NH_3 concentration is influenced by the CP content of seaweed. Getachew et al

(2004) stated that since the truly digested substrate is partitioned among VFA, gas, and microbial biomass, gas metering only account for a substrate that is used for VFA and gas production and do not assume substrate utilized for microbial growth. Satter and Slyter (1974) stated that the NH_3-N concentrations for seaweed were above the level limiting in vitro ruminal microbial growth (5 mg/100 mL).

CONCLUSIONS

It was concluded that brown seaweed (Laminaria sp. and Padina australis) were more degradable than red seaweed (Gracilaria sp. and Eucheuma cottonii) in the rumen. Padina australis is the most potential as ruminant feed because it resulted in the highest propionate proportion, the lowest butyrate and A:P ratio, and low methane production and did not alter the NH3 and microbe protein concentrations.

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AUTHOR CONTRIBUTIONS

Nur Hidayah: Executed the experiment and carried out laboratory analysis, analysed the data, and drafted the manuscript. Kustantinah: Design and supervised the experiment. Cuk Tri Noviandi: supervised the experiment. Andriyani Astuti: Drafted and modified the manuscript. Chusnul Hanim: modified the manuscript, Bambang Suwignyo: modified the manuscript. All authors read and agreed on the final manuscript.

CONFLICT OF INTEREST

All authors declare that they have no competing interests.

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