Enteric methane mitigation by using seaweed *Eucheuma cottonii*

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**Abstract.** Methane is one of greenhouse gasses cause global climate change. Sources of methane emitted by livestock are from enteric fermentation during feed digestion in the rumen of ruminant animals and from manure of ruminant and non-ruminant. Methane enteric contributes the largest portion of total gas emitted by livestock. Therefore, mitigation strategy of enteric methane production is become the important issue. The objective of this study was to determine the effect of *Eucheuma cottonii* (EC) seaweed added to basal diet on enteric methane production and fermentation kinetics by using *in vitro* method. Three level of EC were added to basal diet of Elephant Grass (EG), they were P0 = EG+ 0% EC; P1 = EG + 4% EC; P2 = EG + 8% EC; dan P3 = EG + 12% EC. All treatment was replicate five times. Variables measured were total and methane gas produced, dry matter and organic matter digestibility, pH, and ammonia concentration; rumen protozoa and bacterial population. The data obtained were analyzed using ANOVA and continued with Tukey test. The results showed that increasing level of EC on EG followed by declining methane gas produced by 7.66%; 9.93%; and 16.74%, respectively (P<0.01); increased total bacteria population up to 169.7%, reduced protozoa population up to 69.2%; increased dry matter and organic matter digestibility (P<0.01). It is concluded that supplementation of *E. cottonii* on elephant grass basal diet on dose level of 4%, 8% and 12% reduced CH\(_4\) production as well as increased digestibility of the feed. The best level of *E. cottonii* supplementation in reducing CH\(_4\) production was 12% with can give reduction effect on CH\(_4\) up to 16.74% and increasing on digestibility up to 11.17%.

1. **Introduction**
Methane (CH\(_4\)) gas is a greenhouse gas (GHG) produced mainly by methanogenic microbes that are originate in natural ecosystems including the gastrointestinal tract of ruminant animals [1]. Among the livestock, ruminant animals is the most contributor for GHG who produce about 81 million tons or 29% of total meat production in the world or responsible for about 25% of the total methane emissions in the atmosphere, with beef cattle contribute for about 79% [2,3]. A rapid increase of CH\(_4\) concentration in the atmosphere by more than twofold than in the early 1800s has been reported [4]. In Indonesia, CH\(_4\) enteric from livestock is about 35 million ton CO\(_2\)-eq in year 2012 or increase about 15 million ton CO\(_2\)-eq since 2006 [5]. Moreover the concentration is predicted to become 40 million CO\(_2\)-eq in year 2025 [6].

Ruminant animals produce CH\(_4\) gas as a by-product of the anaerobic microbial fermentation of feeds in the rumen and large intestine [7]. Fermentation of ingested organic matter (OM) by ruminal microbial community, consisted of bacteria, protozoa, fungi, and bacteriophages, produce mainly
volatile fatty acids (VFAs), together with other products such as CO₂, H₂, and formates [8]. All of these end-products of fermentation used by methanogenic archaea present in the rumen to produce CH₄. Most of the CH₄ produced in the rumen is exhaled by the animal, which represents a loss of gross energy intake. This energy loss can be reduced by improved the quality of feed, as reported by [9] that reducing energy loss as CH₄ reached 18.8% and 33.3% when concentrate supplemented to dairy cattle was increased by 30% and 60%. Therefore, developing mitigation strategies that reduce enteric CH₄ formation as well as improving feed utilization, diet digestibility, and increasing livestock productivity is required. The mitigation strategies must be economically viable by improving ruminant productivity.

One of strategies is a management on nutrition offered to the animals. It will be economic because this includes in a short-term strategy in reducing CH₄ emission. Many nutritional strategies have been applied such as increasing in the amount of grain and legumes in the diet, supplementation of feed using oil and fats inhibit formation of methane in the rumen [10–12]. Competition with human need when using grains and supplements to inhibit methanogenesis, triggers to find other alternatives by using dietary additives such as monensin [12] and phenolic compound such as tannins and saponins [13]. However, these compounds reported have variation effect of anti-methanogenic influenced by their molecular structure. Moreover, some compounds also leading to decrease on digestibility [14].

Other alternative is using macroalgae or seaweed, since this material is rich with metabolites essential to metabolic function such as mineral, vitamins, protein, lipids and polysaccharides which can enriched nutrition of feeds and increase lambs growth [15–17]. Some seaweeds reported decrease methane produced in cattle rumen were Gigartina sp, Ulva sp, Saccharina latissima, Laminaria ochroleuca, and Gracilaria vermiculophylla [18]. While, the type of seaweeds potentially produced in seawater Indonesia are Asparagopsis taxiformis and Eucheuma cottonii. The E. cottonii is very easy to cultivate by fisherman, so the production of this seaweed is very abundant. The two seaweed A. taxiformis and E. cottonii contain phycocolloid karaginan, which can reduce CH₄ formation in the rumen. The A. taxiformis reduced CH₄ produced in vitro as well as changed the VFA composition which lead to increase propionate composition [19]. Moreover, [20] reported that supplementation of E. cottonii on basal diet of elephant grass has an effect on VFA composition. The proportion of propionate increased followed by decreasing in acetate proportion. Shifted of VFA proportion from acetate to propionate was estimated to lead CH₄ reduction. However, there is no data available on the effect of E. cottonii supplementation on total gas and gas CH4 production, digestibility and other end-product of fermentation. Therefore the study reported aimed to determine the effect of E. cottonii in ruminal fermentation by in vitro.

### 2. Method

Rumen fluid used in the experiment was collected from two rumen of Ongole cross breed cattle which were fed native grass mixed with rice straw ad libitum. Approximately 500 ml of rumen liquid and solids collected from each animal was mixed, then placed into a thermal flasks. The rumen fluid was blended by using a hand mixer for 30 seconds to ensure a complete mixing between solid and liquid phase [21]. The mixture was strained through a 1 mm mesh, then was continuously purged with high purity of CO₂ gas and placed in waterbath to maintain the temperature at 39°C.

Rumen medium for in vitro determination was prepared using rumen fluid and pre-heated buffer solution [22]. The ratio of rumen fluid and buffer solution was 1:4 (v:v). The feed samples of elephant grass and seaweed E. cottonii were prepared by oven dry at 60°C for 3 days, then ground through 1 mm sieve. Nutrients content of elephant grass and E. cottonii are presented in table 1.

| Table 1. Nutrient content of feed and seaweed used in the experiment. |
|------------------|-------|-------|--------|--------|------|---------|
|                  | Ash (%) | Crude protein (%) | Crude fiber (%) | NDF (%) | Fat (%) | Gross energy (Kcal/kg) |
| Elephant grass   | 10.8 | 9.7 | 31.7 | 70.18 | 2.3 | 4739 |
| Eucheuma cottonii | 14.39 | 5.33 | 2.77 | - | 1.03 | 1554 |
The *in vitro* was conducted following completely randomized design using a series of batch culture incubations following the procedure of [22], in order to determine the effect of addition seaweed *E. cottonii* on ruminal fermentation of elephant grass feed i.e: total gas and CH$_4$ gas production, dry matter (DM) and organic matter (OM) digestibility, ammonia (NH$_3$) concentration and total population of bacteria and protozoa. Feed samples containing mixture of elephant grass and *E. cottonii* with three different ratios as treatments, were weighed into 150 ml incubation bottle. One gram of dry feed sample, following the treatments, were weighed into 150 mL incubation bottles. The anaerobic conditions of the bottles were optimized by purged with CO$_2$ gas, sealed and incubated at 39°C in temperature controlled waterbath. The four treatments were 1) a positive control bottle containing 1 g of elephant grass (P0); 2) treatment 1 was 96% elephant grass and 4% *E. cottonii* in dry bases (P1); 3) treatment 2 was 92% elephant grass and 8% *E. cottonii* in dry bases (P2); 4) treatment 3 was 88% elephant grass and 12% *E. cottonii* in dry bases (P3). Five replicates were applied for each treatment, and three bottles for blank containing only rumen medium were also included in waterbath.

All the bottles was monitored for 48 h with reading intervals for gas total and CH$_4$ gas was on 2, 6, 12, 18, 24, 36, and 48 hours to generate total gas production curves. Gas was measured by using 50 mL glass syringe attached with a needle. At each monitoring time, measurement of gas produced in each bottle was conducted by inserting a needle equipped with a glass syringe into the bottle. The gas in the incubator bottle flowed into the glass syringe. The amount of gas stored in the syringe was then recorded as the total gas produced. Gas CH$_4$ produced was measured by following procedure of [23]. Total gas collected was then flowed through a tube connected to the Erlenmeyer containing NaOH 6N solution. Other gases besides methane gas will be bound by the solution, so that the CH$_4$ gas present flowed and be measured in another glass syringe connected at the end of the circuit.

After 48 h incubation, pH of rumen medium was measured using pH meter. Ammonia concentration was measured by analyzing the residual fluids using Micro diffusion Conway method [24]. Solid residues were analyzed for apparent degradability of dry matter (DMD) and organic matter (OMD). Total bacteria and protozoa population were analyzed from residual fluids following roll tube methods as described by [25].

One-way analyses of variance (ANOVA) were used to compare the differences in variables measured between treatments. Post-hoc comparisons were made using Tukey's test. All analysis was conducted using software statistics SPSS 22.

### 3. Results and discussion

#### 3.1. Total gas and CH$_4$ production

The total gas production (TGP) and gas production rate (GPR) are shown in figure 1. The TGP pattern for P0 and P3 group was above the TGP pattern for P1 and P2 group for whole incubation time (48 hours). These patterns indicated that treatment P0 and P3 might have similar TGP, they were higher than those of TGP in treatments P1 and P2.

![Figure 1. Total gas production (a) and gas production rate (b) of four treatments over 48 hours incubation.](image-url)
The study also found a different pattern of GPR among the four treatment groups during the first 12 hours of incubation, but similar pattern for all treatments groups for the next 24 hours of incubation time, then following with different pattern again between P0 and P1 vs P2 and P3 during the last 12 hours of incubation time. During the first 4 hours of incubation time, gas produced in group treatment P3 was higher than others, then followed by gas produced in group treatments P0, P2 and P1. Meanwhile during the last 12 hours of incubation time, group treatment P2 and P3 still produce higher gas than those of group treatments P0 and P1, which indicated more OM digested during this period. The result indicated that feed in treatment P3 was easy and readily to degraded in the rumen followed by feed in treatment P0, P2 and P1. Feed in treatment P3 contains more E. cottonii (12%) compared to those in P2 (8%) and P1 (4%). As shown in table 2, TGP of P3 was significantly higher than the other three groups, followed by P2, P0 and P1 groups (P<0.01).

The TGP has correlation with the amount of OM digested by rumen microbes. The study indicated that increasing in level of E. cottonii supplementation resulted an increasing in TGP and more DM and OM digested (P<0.01). In term of methane gas production, supplementation of E. cottonii strongly affect the amount of gas CH4 produced (P<0.01). Addition of E. cottonii reduced the amount of gas CH4 produced, as shown in the percentage of gas CH4 produced per TGP produced, which indicated that there were significant declining by 7.66%; 9.93%; and 16.74% on group P1, P2 and P3, respectively (P<0.01). Among the three levels of supplementation, the declining was similar between P1 and P2, but those two groups were different with that of P3 group (table 2).

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### Table 2. Average of gas production and digestibility of feed examined in the study.

| Variables                          | P0                  | P1                  | P2                  | P3                  |
|------------------------------------|---------------------|---------------------|---------------------|---------------------|
| Total gas production (ml)          | 143.20±1.47<sup>b</sup> | 129.00±0.89<sup>d</sup> | 133.20±3.31<sup>c</sup> | 148.20±2.23<sup>a</sup> |
| Gas CH<sub>4</sub> production (ml) | 20.20±0.51<sup>a</sup> | 16.80±0.24<sup>b</sup> | 16.90±0.20<sup>b</sup> | 17.40±0.37<sup>b</sup> |
| Gas CH<sub>4</sub> per total gas (%) | 14.10±0.27<sup>b</sup> | 13.02±0.21<sup>b</sup> | 12.70±0.43<sup>b</sup> | 11.74±0.23<sup>b</sup> |
| Dry matter digestibility (%)       | 50.04±1.13<sup>b</sup> | 54.50±0.39<sup>a</sup> | 54.80±1.47<sup>a</sup> | 55.34±0.86<sup>c</sup> |
| Organic matter digestibility (%)  | 50.69±1.55<sup>b</sup> | 54.67±0.82<sup>a</sup> | 55.68±2.37<sup>a</sup> | 56.35±1.15<sup>a</sup> |
| Total gas per unit OM digested (ml/g OMD) | 0.283±0.006<sup>a</sup> | 0.237±0.004<sup>c</sup> | 0.240±0.011<sup>c</sup> | 0.260±0.01<sup>b</sup> |
| Gas CH<sub>4</sub> per unit OM digested (ml/g OMD) | 0.040±0.001<sup>a</sup> | 0.031±0.001<sup>b</sup> | 0.030±0.001<sup>b</sup> | 0.030±0.001<sup>b</sup> |

Notes: P0 : Elephant grass 100% (Control) ; P1: Elephant grass 96% + E cottonii 4%; Elephant grass 92% + E cottonii 8% and Elephant grass 88% + E. cottonii 12%. Different superscript in the same row shows significantly different (P<0.01).

All treatments group of E. cottonii supplementation (P1, P2 and P3) has higher dry matter (DM) and organic matter (OM) digestibility than those of control group (P0) (P<0.01). There were an increasing on DM and OM digestibility by 9% and 7.9%; 9.5% and 9.8%; 10.6% and 11.17%, for P1, P2 and P3 group respectively. When CH4 gas production expressed per unit of OM digested, supplementation of E. cottonii significantly reduced TGP and CH4 gas produced per unit of OM digested (P<0.01). Among the three treatment groups, P1 and P2 have similar values of CH4 gas produced per unit of OM.

3.2. End-product of fermentation

The results for other end-product fermentation of the four feeds examined in the study are presented in table 3. The study indicated that there were similar value of ammonia concentration and fermentation pH among the four groups. Although the value was vary among the four groups, they were not significant different (P>0.05). The finding also indicated that there were significant differences on total bacteria and protozoa population among the four groups (table 3). Total bacteria population was significantly higher on P3 group, then followed by group P2, P1 and P0 (P<0.01). However, the reverses pattern was recorded for total population of protozoa, the highest population was found on P0 groups (P<0.01), followed by P1, P2 and P3 groups.
**Table 3.** Average of pH, Ammonia, bacteria and protozoa population determined from feed examined in the study.

| Variables                        | P0       | P1       | P2       | P3       |
|----------------------------------|----------|----------|----------|----------|
| pH                               | 6.87±0.10 | 6.88±0.10 | 6.92±0.04 | 6.97±0.05 |
| Ammonia concentration (mg/L)     | 371.96±20.01 | 348.16±15.60 | 338.64±16.60 | 334.56±23.81 |
| Total bacteria (x 10^7 cel/mL)   | 5.23±0.54⁴  | 7.65±0.24³  | 11.64±0.56²  | 14.10±0.23³  |
| Total protozoa (x 10^³ cel/mL)   | 64.30±0.54⁴  | 42.60±1.24³  | 32.80±2.98²  | 19.80±2.14³  |

Notes: P0 : Elephant grass 100% (Control) ; P1: Elephant grass 96% + E. cottonii 4%; Elephant grass 92% + E. cottonii 8% and Elephant grass 88% + E. cottonii 12%. Different superscript in the same row shows significant different (P<0.01).

There were correlation between digestibility and total population bacteria. The highest number of bacteria present in the fermentation system of P3 was followed by the most digested of DM and OM of the feed on P3. In reverse side, the lowest bacteria population on P0 resulted in the less DM and OM digested in the fermentation system.

The study indicate that there were consistence effect of E. cottonii addition on in vitro fermentation, with the effect was level E. cottonii dependent. Feed digestibility was influenced by nutrient content of the feed. Feed contains more fiber and NDF digested in a low level than that of feed with less crude fiber and NDF content [26]. In the current study, seaweed E. cottonii contains low crude fiber and no NDF, therefore addition of E. cottonii up to 12% on dry matter base on elephant grass diet reduce crude fiber and NDF content of the rations. In consequence resulted on higher digestibility values compared to that of elephant grass without addition of E. cottonii. The NDF content also has an effect on CH4 gas produce. Finding in this study was coherent with study by [27], which reported that the increasing on NDF content on the diet resulted on increasing on CH4 gas produced, then followed by low digestibility value. Supplementation of elephant grass by E. cottonii reduced the content of NDF on the feed examined, thus declined the CH4 gas produced.

The current findings also comparable with that study by [20], who reported that supplementation of E. cottonii was estimated reduce CH4 production when it was calculated from acetate and propionate composition resulted from in vitro fermentation. Supplementation of elephant grass with E. cottonii increase total VFA concentration, with an increasing in proportion of propionate produced. Shifted of acetate to propionate proportion leads to less CH4 formation during fermentation of feed by rumen microbes.

The effect of reduction CH4 production when E. cottonii added to elephant grass basal diet was not only caused by the nutrient content of the feed (less fiber and NDF content), but it might also due to the present of substance with has a potential as antimethanogenic effect. As reported that many macroalgae (red, brown and green) contain lipid, tannin and secondary compound with functional as antimethanogenic [28,29]. Although the effect were varied among the type of macroalgae, the place where the macroalgae harvested, and also the dose level of macroalgae supplemented. Further study related to the secondary compound contain, the type of secondary compound and its effect on CH4 gas production might be required.

With this potential in reducing CH4 production as well as increase the digestibility value of basal diet, seaweed E. cottonii promising to be used as feed additive for ruminant animals. Since the cultivation of this seaweed is easy, so many fisherman in Indonesia can cultivate this seaweed. Commercialization of this seaweed as feed additive for ruminant animals can have economically benefit for fisherman to increase their income.

4. Conclusion
Seaweed E. cottonii potential in reducing CH4 formation in in vitro fermentation. Supplementation of E. cottonii on elephant grass basal diet on dose level of 4%, 8% and 12% reduced CH4 production as well as increased digestibility of the feed. The best level of E. cottonii supplementation in reducing CH4 production was 12% with can give reduction effect on CH4 up to 16.74% and increasing on digestibility up to 11.17%. 

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