The effect of seaweed (Gracilaria sp.) supplementation in sheep feed on methanogenesis inhibition in vitro

Caribu Hadi Prayitno, Faby Kurnia Utami, Adi Nugroho, and Titin Widyastuti
Animal Science Faculty, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.
Email: caribu_prayitno@yahoo.co.id

Abstract. The study aimed to assess the optimal level of supplementation of seaweed Gracilaria sp. in sheep feed on total gas, methane gas, methanogen and protozoa populations, digestibility of dry matter and organic matter. The research material used was rumen fluid from 3 sheep. The treatment tested was supplementation of seaweed flour Gracilaria sp. with a level of 0% (P0), 2% (P1), 4% (P2), 6% (P3) and 8% (P4) based on feed DM which was composed of forage Cynodon dactylon (60%) and concentrate (40%). The study design used a completely randomized design, each treatment was repeated 4 times. The variables measured are total gas, methane gas, methanogen population, protozoa population, dry matter and organic matter digestibility. The results showed that the supplementation of Gracilaria sp. seaweed decreased (P<0.05) total gas, methane gas, methanogen population, protozoa population and increased (P<0.05) digestibility of dry matter and organic matter. Conclusions, seaweed supplementation up to the level of 3.6% (DM) effective inhibited the level of methanogenesis giving varying influences.

1. Introduction
Indonesia is a country that is mostly filled with oceans. This also supports the availability of diverse seaweed, one of which is red seaweed, namely Gracilaria sp. Extensive distribution of Gracilaria sp. in Indonesia it reaches 255 km² [1]. Gracilaria sp. is a type of red seaweed that grows in Indonesian waters. Gracilaria sp. resistant to the influence of fresh water, can even live in brackish water, so many types of Gracilaria sp. found on the beaches of large islands.

Gracilaria sp. is a type of seaweed that has halogen compounds, especially bromoform, saponin, tannin. Halogen compounds, bromoform are anti-methanogen compounds, which will react with vitamin B12, thus inhibiting the ability of enzymes from methanogens in the formation of methane gas [2]. The saponin compound is a defaunation agent for protozoa, so it can reduce the population of protozoa in the rumen fluid thereby increasing digestive activity in the rumen. Condensed tannins are compounds that can protect proteins so that they can improve post-rumen digestion and improve feed digestibility.

2. Research methods
The study used an experimental method in vitro according to [3] modified by [4]. The material used is rumen fluid derived from 3 sheep that have been adapted to basal feed supplemented with grass. Sheep feed consisted of forage Cynodon dactylon (60%), concentrate (40%) and seaweed flour (Gracilaria sp.) with the composition of the treatment ration shown in table 1.
Table 1. Ration composition

| Feedstuff          | P0 | P1 | P2 | P3 | P4 |
|--------------------|----|----|----|----|----|
| Field grass        | 60 | 60 | 60 | 60 | 60 |
| Concentrate        | 40 | 40 | 40 | 40 | 40 |
| - Cassava by product | 47.5 | 47.5 | 47.5 | 47.5 | 47.5 |
| - Pollard          | 25 | 25 | 25 | 25 | 25 |
| - Coconut meal     | 7.72 | 7.72 | 7.72 | 7.72 | 7.72 |
| - Palm cake        | 11 | 11 | 11 | 11 | 11 |
| - Soybean Meal     | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 |
| - Dolomit          | 1.43 | 1.43 | 1.43 | 1.43 | 1.43 |
| - Salt             | 1  | 1  | 1  | 1  | 1  |
| - Molases          | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| - Mineral          | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 |
| Gracilaria sp.     | 0  | 2  | 4  | 6  | 8  |
| **Total**          | 100 | 102 | 104 | 106 | 108 |

Description: P0: control feed, P1: P0 + seaweed flour (*Gracilaria sp.*) 2%, P2: P0 + seaweed flour (*Gracilaria sp.*) 4%, P3: seaweed flour (*Gracilaria sp.*) 6%, P4: seaweed flour (*Gracilaria sp.*) 8% of DM.

The research design used was a completely randomized design with 5 treatments, each treatment was repeated 4 times so that there were 20 experimental units. The test of variance analysis stated that the treatment had a very significant effect (P <0.01) followed by polynomial orthogonal test to see the response curve [5]. There are 5 treatments, namely:

P0 = Rumen liquid + basal feed (PK = 11.8%; TDN = 60%)
P1 = P0 + seaweed flour *Gracilaria sp.* 2%
P2 = P0 + seaweed flour *Gracilaria sp.* 4%
P3 = P0 + seaweed flour *Gracilaria sp.* 6%
P4 = P0 + seaweed flour *Gracilaria sp.* 8%

The variables measured in the study were feed digestibility, total gas, methane gas, methanogen population with roll tube method [6] and protozoa population [7].

3. Results and discussion

3.1 Effect of treatment on total gas concentration

The results of this study showed that supplementation of 2% seaweed observed at 0, 2, 4, 6, 10, 14, 22, 24, 30, 36, 48 hours was able to reduce total gas by 43.66%. The total gas in the rumen fermentation process consists of CO2, H2, and CH4. This total gas reduction shows an inhibition of the activity of gas-producing microbes.

3.2 Effect of treatment on methane gas concentration

The results of this study showed that supplementation of 2% seaweed observed at 0, 2, 4, 6, 10, 14, 22, 24, 30, 36, 48 hours was able to reduce methane gas by 49.0%, increasing level up to 6% will reduce the concentration of methane but levels above 6% have not affected methane gas. The decrease in methane gas is thought to have inhibited methanogen in producing methane gas, or there has been a decrease in gas supply of CO2 and H2 from the main fibrolytic microbes that produce CO2 and H2 gas.
3.3 Effect of treatment on methanogen populations

The results showed the average methanogen population listed in table 2.

| Treatment | Average of Methanogen population (CFU/ml) | Decreased Methanogen Population (%) |
|-----------|------------------------------------------|-------------------------------------|
| P0        | 43.750 ± 6.849,57                        | -                                   |
| P1        | 13.500 ± 4.725,82                        | 69                                  |
| P2        | 7.000 ± 2.581,99                         | 84                                  |
| P3        | 31.500 ± 5.742,7                         | 25                                  |
| P4        | 18.500 ± 3.269,32                        | 58                                  |

The results showed that the methanogen population had the highest decrease in the level of addition of 4% (P2) with a population of 7,000 CFU / ml, and the lowest decrease in addition of 6% (P3) with a population of 31,500. The effect of giving at the 4% level is close to the results of research by [2], that with the addition of 5% red seaweed as *Asparagopsis taxiformis* can reduce the total production of methane gas by 50%. Methane gas has decreased due to the reduced methanogen population. The
results showed that the supplementation of *Gracilaria sp*. seaweed flour very significant effect (P <0.01) on the methanogen population. The results of orthogonal polynomial test show the quartic responses shown in figure 3.

**Figure 3.** Graph of the response of methanogen populations

Based on the graph, the equation $Y = -197.27X^4 + 2518.2X^3 - 6617.2X^2 - 10385X + 43750$, with a coefficient of determination (R²) of 78.33%. Based on these results, the minimum point is obtained at the level of supplementation of *Gracilaria sp*. Seaweed flour. 3.3% with a methanogen population of 4,520 CFU / ml, and the maximum point at the level of addition is 6.8% with the population of methanogens as much as 37,165 CFU / ml. Bromoform compounds found in *Gracilaria sp.* is the cause of the decline in the methanogen population. Bromoform is a compound that functions to inhibit the work of methanogens specifically. This was explained by [8] that bromochloromethane would reduce the activity of coenzyme cobalamin (vit. B12) and coenzyme methyl M-reductase (mcrA) which is in methanogen DNA. The decrease in the population of methanogens in the rumen fluid is expected not to affect the fermentation results in the digestive tract, especially the rumen.

### 3.4 Effect of treatment on protozoa population

The results showed the average rumen protozoan population shown in table 3.

| Treatments | Protozoa Population (cell/ml) | Decreased of Population (%) |
|------------|-------------------------------|----------------------------|
| P0         | 15.450 ± 2.289,83             | -                          |
| P1         | 9.700 ± 1.336,66              | 37                         |
| P2         | 7.265,5 ± 1574,85             | 53                         |
| P3         | 11.525 ± 2.617,09             | 25                         |
| P4         | 7.656,25 ± 1.457,51           | 50                         |

Based on the results of the study, the lowest population of protozoa was found in the third treatment (P2) which was equal to 7,266 cells / ml, with the supplementation of *Gracilaria sp*. Seaweed flour as much as 4% of the total dry matter of feed, and the highest population of protozoa was in the fourth treatment (P3) other than control which was 11,525 cells / ml with the addition of *Gracilaria sp*. seaweed flour by 6%.
Based on the graph, the quartic response curve is obtained and the equation \( Y = -47.398X^4 + 639.16X^3 - 2093.4X^2 - 865.7X + 15450 \), with the coefficient of determination (R^2) of 77.41%. The results of Orthogonal Polynomial test show that the minimum point in the response chart is at the 3.6% point of addition of *Gracilaria sp.* flour with a population of 7,063 cell / ml protozoa, and the maximum point at the level of 6.7% addition of *Gracilaria sp.* with a population of 12,400 cells / ml protozoa. Tannin and saponin compounds contained in *Gracilaria sp.* participate in decreasing the protozoan population.

3.5 Effect of treatment on dry matter and organic matter digestibility

The results showed that the digestibility of dry matter and organic matter in each treatment as in table 4.

### Table 4. Average of digestibility level

| No | Treatments | Dry Matter Digestibility (%) | Organic Matter Digestibility (%) |
|----|------------|-----------------------------|---------------------------------|
| P0 | 0%         | 65.8 ± 1.53                 | 63.96 ± 2.22                    |
| P1 | 2%         | 63.42 ± 0.92                | 60.17 ± 1.69                    |
| P2 | 4%         | 66.29 ± 1.37                | 65.69 ± 3.29                    |
| P3 | 6%         | 69.35 ± 1.4                 | 68.33 ± 1.61                    |
| P4 | 8%         | 71.04 ± 2.44                | 69.72 ± 2.77                    |

Feed digestibility is the amount of feed substances that can be digested during the digestive process in the digestive tract. The results showed that the higher the supplementation of *Gracilaria sp.* flour, on feed able to increase feed digestibility level, shown in table 3. The highest average yield was at the level of 8% for dry matter digestibility of 71.04% and organic matter digestibility of 69.72%. The lowest digestibility values were at the level of addition of 2%, namely 63.42% for dry matter digestibility and 60.17% for digestibility of organic matter.
Based on this equation, the highest dry matter digestibility value of feed is at the level of 8% \textit{Gracilaria sp} flour with the digestibility percentage of 71.02%. The lowest value of dry matter digestibility at the grading rate of 1.8% with a percentage of 63.3% was thought to be due to the level of administration of 1.8%. The highest value of organic matter digestibility is at the level of 8% giving 69.76% and the lowest at the level of 1.7% at 59.67%. The \textit{Asparagopsis taxiformis} red seaweed would have the effect of increasing propionate production with a minimum of 2% [2]. Giving a minimum of 2% \textit{Asparagopsis taxiformis} shows that at this level the bioactive compounds contained begin to have an impact on the condition of the rumen microflora. Increased value of feed digestibility, due to the presence of saponins and tannins in \textit{Gracilaria sp}. Saponins are able to reduce the population of protozoa in the rumen [9,10], which stated that the population of rumen protozoa decreased due to tea pulp supplementation in feed containing saponins of 0.4–1.2%. The decrease in protozoa population resulted in an increase in the total cellulolytic and amylolytic bacteria and the digestibility level of feed dry matter. Feed fermentation in the rumen will increase with increasing numbers of feed digestive bacteria.
4. Conclusion
Supplementation of *Gracilaria sp.* in sheep feed to the level of 3.6% from feed DM effectively inhibits methanogenesis. Increased levels of up to 8% can increase feed digestibility but provide less tangible effects in inhibiting methanogenesis.

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