The Effect of Fitosan Supplementation on Methane Production in Cow’s Rumen Liquid by In Vitro Method

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Abstract. Methane is one of greenhouse emission gas causing climatic change and global warming. The biggest source of methane comes from the livestock sector. The objective of this study was to investigate the effects of fitosan on methane production by using in vitro method. Before the in vitro test, the antimicrobial test of fitosan was measured by looking at the clear zone of the media containing bacteria Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative). In vitro method should be done by adding fitosan (0.15 mL, 0.3 mL, and 0.6 mL/30 mL media) and grass field powder into cow’s rumen liquid. Based on the results, fitosan has an antimicrobial ability to decrease the microbial activities both of Gram-positive or Gram-negative. Testing with the in vitro method showed that fitosan have the potency to reduce methane gas production about 23.34 – 31.85% compared to controls (without fitosan). The best treatment is treatment with the addition of fitosan with concentration of 0.6 mL per 30 mL of rumen fluid.

Key words: Fitosan, Cow’s Rumen Liquid, Methane Production, In vitro.

1. Introduction
Global warming is caused by an increase in greenhouse gas (GHG) emissions, including carbon dioxide (CO₂) and methane gas (CH₄). Methane gas is the most dangerous GHG and has increased about the concentration of 0.9% per year in the atmosphere [1]. CH₄ is present in atmosphere very low compared CO₂ but it is 21 times more potent per unit as a GHG. The GHG emissions from the agricultural sector account for about 25.5% of total global anthropogenic emission. Globally, the ruminant livestock sector is a major source of anthropogenic (human-induced causes) methane emission with annual global contribution of 14.5 percent [2].

Methane from the livestock sector is produced in the digestive tract. In ruminants, 80-95% of methane gas is produced in the rumen and 5-20% is produced in the large intestine. Methane gas is produced in the fermentation process of microorganisms in the digestive system of ruminants which will be released through the process of eructation. High methane gas is not only harmful to the environment but also harms the animal itself. Livestock can lose up to ± 12% of total chemical energy,
thereby reducing the efficiency of digestion [2]. The strategy that can be used to reduce methane gas emissions in livestock digestion is by modifying the feed given [4].

Alternative supplement that can be used to suppress methane H4 gas are Fitosan. It’s a research product of the National Nuclear Energy Agency (BATAN) which is a chitosan-processed product that has been irradiated with the dose of 75 kGy [5]. Chitosan is a polysaccharide prepared by deacetylation of chitin which is widely distributed in living organisms such as crustacea, insects, and fungi [6,7]. Gamma irradiation dose has resulted the low molecular weight chitosan (MW 14 kDa). The results showed that gamma irradiation is an effective method of degrading chitosan by cleavages of glycosidic bond. Fitosan has been used extensively in the agricultural sector [5]. In the U.S., chitosan has been deemed generally recognized as safe (GRAS), which ultimately allows for chitosan to be used as an alternative to antibiotics [8].

It was reported previously that dietary supplementation of chitosan was able to improve animal growth performance [9,10], while other studies claimed the opposite [11,12]. Henry et al. [13] showed that the addition of chitosan to cattle rumen fluid can improve nutrient digestibility even though it does not significantly reduce the methane gas emissions produced. In addition, the results of research conducted by Goiri et al. [8] showed that the addition of chitosan shortens the time of adaptation of rumen fluid when tested in vitro. Chitosan supplementation to grazing steers positively affected DM intake and digestibility, increased ruminal propionate concentration and calculated microbial protein production [14].

The research about supplementation fitosan for ruminants has not been conducted. Therefore, the objective of this study was to determine the effects of fitosan on fermentation product and methane production from cow rumen fluid by using in vitro method. Before the in vitro test, fitosan antimicrobial testing of Gram-negative and positive bacteria was tested to determine its potential in inhibiting microbial growth of rumen fluid.

2. Methods

2.1. Antimicrobial Test

The cultures of E. coli (Gram-) and S. aureus (Gram +) were grown on 30 mL Nutrient Broth medium for 24 h at room temperature and agitation 120 rpm. Then 0.1 mL culture (A660nm=1) was poured into a plate and mix with Nutrient Agar Media. After freezing, 250 and 500 µL fitosan was added to the agar surface. The clear zone was observed after 24 h incubation.

2.2. In Vitro Test

Before the in vitro rumen test, field grass was dried for 4 days in an incubator at 55 °C and grinding with a size of 100 mesh. The study was conducted with four treatments i.e. F1 (addition of 0.15 mL fitosan), F2 (0.3 mL) and F3 (0.6 mL), also control (without fitosan). For in vitro gas production test, 0.2 g field grass and fitosan was added with 30 mL medium (rumen liquid and McDougall’s solution) in 100 mL capacity Hohenheim gas syringe and incubated at 39°C for 24 h. Total gas production was calculated as the displacement of the piston in 24 h [15]. After 24 h, rumen liquid was measured for pH, ammonia with Conway method, and VFA with distillation method. Data were subjected to analysis by ANOVA and means were compared using Duncan’s multiple range test. All statistical analysis was performed using SPPS (20.0 version) computer package.

3. Results and Discussion

Antimicrobial testing showed that fitosan was able to inhibit the growth of Gram - (E. coli) and + (S. aureus) bacteria with different clear zone diameters (Table 1). The diameter of the clear zone formed is proportional to the concentration of fitosan given. Antimicrobial activity in Gram-negative bacteria was higher than Gram-positive. The presence of antimicrobial ability indicates that fitosan has the potential to inhibit bacterial growth in rumen fluid and will affect its metabolic activity. Gram-negative bacteria in rumen fluid, generally play a role in the metabolism of carbohydrate, protein and
fat decomposition, while Gram - bacteria play a role in the process of methanogenesis or production of methane [4].

| Fitosan (mL) | Diameter of clear zone (cm) | Escherichia coli | Staphylococcus aureus |
|-------------|-----------------------------|----------------|-----------------------|
| 0.25        |                            | 1.5 ± 0.2       | 1.3 ± 0.3             |
| 0.5         |                            | 2.2 ± 0.1       | 2.1 ± 0.2             |

Although fitosan has the antimicrobial ability, the results of in vitro tests indicate microbial activity (Table 2). This is indicated by the change in pH value, the concentration of ammonia and VFA and the number of protozoa. The initial pH of the rumen fluid has decreased after the addition of fitosan. After 24 hours of incubation, the pH value of all treatments and controls increased. The pH value is still in the normal pH range for rumen microbial activity, the initial pH of the rumen fluid decreases after the addition of fitosan. After 24 hours of incubation, the pH value of all treatments and controls increased. The pH value is still in the normal pH range for rumen microbial activity, about 5.5 – 7.6 [16]. Addition of fitosan did not cause inhibition of the degradation process of carbohydrate and protein compounds. This is marked by an increase in the concentration of ammonia and VFA. Ammonia is a result of decomposition of proteins, while VFA is a decomposition of carbohydrates [17]. Fitosan supplementation proved to inhibit the growth of protozoa. This condition can be a potential for a potential decline in methane gas production. In the body of the protozoa, there are methanogenic microbes. Both have a symbiosis that is mutually beneficial (mutualism) [18,19]. Fitosan is the same as chitosan, has properties as an antibiotic so it can inhibit the growth of microorganisms [13].

| Treatments | Times (h) | pH   | Ammonia (mM) | VFA (mM) | Protozoa (cells/mL) |
|------------|-----------|------|--------------|----------|---------------------|
| F1         | 0         | 7.16 | 3.85         | 1.29     | 1.0 x 10^7          |
|            | 24        | 7.45 | 4.12         | 1.34     | 9.3 x 10^6          |
| F2         | 0         | 7.26 | 3.75         | 1.32     | 1.0 x 10^7          |
|            | 24        | 7.41 | 4.02         | 1.42     | 8.2 x 10^6          |
| F3         | 0         | 7.27 | 4.01         | 1.44     | 1.0 x 10^7          |
|            | 24        | 7.29 | 4.13         | 1.52     | 7.5 x 10^6          |
| Control    | 0         | 7.33 | 3.65         | 1.44     | 1.0 x 10^7          |
|            | 24        | 7.36 | 4.11         | 1.65     | 1.2 x 10^7          |

Note: F1: 15 mL fitosan; F2 : 0.3 mL; F3 : 0.6 mL; control (without fitosan)

Methane gas production after addition of fitosan has decreased (Figure 1). Fitosan have potency to reduce methane gas production about 18.92 - 47.02% compared to controls (without fitosan). The best treatment is treatment with the addition of fitosan by 0.6 mL per 30 mL of rumen fluid. This occurs because fitosan contains polyphenols [14] and can suppress the growth of protozoa which are hosts of methanogens. Some studies have shown the same results. that chitosan affected the ruminal fermentation and reduced methane production of rumen fluid by in vitro method [8,20]. Other research said that enteric methane emissions were not affected by chitosan supplementation [11-13].
4. Conclusions
Fitosan has an antimicrobial ability to decrease the microbial activities both of Gram + or Gram -. Testing with the in vitro method showed that fitosan have the potency to reduce methane gas production about 23.34 – 31.85% compared to controls (without fitosan). The best treatment is treatment with the addition of fitosan by 0.6 mL per 30 mL of rumen fluid.

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