Reduction in Methane Productions from Sheep by Supplemented of *Moringa oleifera* Leaf Extract in The Ration

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Abstrak. Suatu penelitian yang bertujuan untuk menurunkan produksi gas metan domba melalui penambahan ekstrak daun *Moringa oleifera* telah dilaksanakan menggunakan Rancangan Acak Lengkap (RAL), *One Way Classification*. Sebagai perlakuan yaitu ekstrak daun *Moringa oleifera* dengan taraf 0%, 0,25%, dan 0,50% dari bahan kering pakan, setiap perlakuan diulang enam kali sehingga terdapat 18-unit percobaan. Materi yang digunakan yaitu cairan rumen yang berasal dari tiga domba ekor tipis yang diambil dari Rumah Potong Hewan Banyumas Jawa Tengah segera setelah domba dipotong. Daun kelor dikeringkan dalam oven suhu 60ºC selama 2 x 24 jam, kemudian dibuat tepung dan diekstraksi menggunakan etanol. Pakan terdiri dari kosentrat dan jerami padi amoniasi dengan imbangan 60:40 berdasarkan bahan kering pakan. Konsentrat terdiri dari dua bagian dedak padi dan satu bagian bungkil kelapa. Variabel yang diukur terdiri dari jumlah protozoa, produksi gas metan, jumlah bakteri, konsentrasi N-NH_3 dan sintesis protein mikroba rumen. Hasil penelitian yang diperoleh yaitu jumlah protozoa, produksi gas metan terendah, dan jumlah bakteri, sintesis protein mikroba tertinggi dicapai oleh cairan rumen yang mendapat tambahan ekstrak daun *Moringa oleifera* 0,50% (berdasarkan BK pakan).

Kata kunci: amonia, bakteri, metan, *Moringa oleifera*, protozoa, protein mikroba

Abstract. This study aimed to evaluate the decreasing methane gas production in sheep by supplementing *Moringa oleifera* extract in the ration. The study was conducted in a Completely Randomized Design with One Way Classification. The treatment of *Moringa oleifera* extract was offered at 0, 0.25 and 0.50% of DM ration, each with six replicates, which constitutes 18 treatment units. The materials included ruminal fluid of three freshly slaughtered thin-tailed sheep derived from an abattoir in Banyumas Central Java. *Moringa oleifera* leaves were oven-dried at 60ºC for 2 x 24 h, grounded into powder and extracted using ethanol. The ration consisted of concentrate and ammoniated rice straw with a ratio of 60:40 (DM basis). The concentrate was composed of 2:1 rice bran and cocconut meal. The measured variables included total protozoa, methane gas production, total bacteria, N-NH_3 concentration, and protein synthesis of ruminal fluid. The result indicated 0.50% *Moringa oleifera* extract (DM basis) produced the lowest total protozoa, the highest total bacteria and protein microbe synthesis, and the lowest methane gas production.

Key words: ammonia, bacteria, methane, *Moringa oleifera*, protozoa, microbial protein

Introduction

Smallholder livestock farming in Indonesia is dominated with a traditional management system which offers low-quality feed (farming waste) such as rice straw with low nutrient content, especially the fermentable carbohydrate. Also, rice straw contains low crude protein (5.06%) and high lignin which can bind cellulose to form lignocellulose; consequently, the cellulose is difficult to digest in the rumen (Herawati and Adang, 2010). Therefore, rice straw-based feed would trouble energy and protein fulfilment for rumen microbes and the animal host.

In low-quality feed, protozoa will consume bacteria and convert protein into protein protozoa. As a result, the bacteria population decreases but protozoa population grows, and it reduces protein supply for the animal host. Also, the growing protozoa population would increase methane gas production by 9-25% because methanogen bacteria form a symbiosis by attaching themselves to the surface of protozoa (Santoso and Hariadi, 2007).

The formation of methane gas in the rumen affects the final fermentation product, especially total ATP mol, which also affects the efficiency of rumen microbe production (Thalib, 2008). Ruminants produce methane gas (CH_4) that contributes to the accumulation of greenhouse gas in the atmosphere. Kreuzer and Soliva (2008) reported that methane gas from ruminants supplies 95% of total methane emission from human and cattle and 18% of total greenhouse gas in the atmosphere. The methane emission is related to environmental problems, and it reflects the loss of half energy from cattle for production. It is reported that there was 6-10% loss of gross energy in the form of methane (Jayanegara and Sofyan, 2008). Accordingly, it is important to formulate a feeding strategy to reduce methane emission in ruminant livestock that could reduce the accumulating rate of greenhouse gas (long-term) and decrease the loss of cattle energy (short-term).

Some techniques to limit CH_4 gas production in cattle include the use of chemicals such as monesin, ∝-bromoethanesulfonic acid, and nitrate/nitrite. However, prolonged use of high-concentrate chemicals could produce a residue in cattle products which renders toxic effects on cattle; therefore, the additives substance is not recommended to control CH_4 production (Haque, 2018). Recently, natural feed additives have been replacing the chemicals such as antibiotics and ionophore as the fermentation manipulator in rumen. Susanti and Marhaeniyanto (2014) reported that saponin from plant, such as *Moringa oleifera* could reduce methane gas production.

Plant-derived saponin could improve the efficiency of fermentation process through the mechanism of reducing protozoa population in the rumen by diminishing predatory properties in protozoa against bacteria (Ramos-Morales et al. 2017). The decreasing protozoa population in the rumen has increased bacteria population and reduced protein turnover in the rumen; therefore, it increased total microbial protein entering duodenum (Hess et al., 2004). Saponin is toxic for protozoa, and 9-25% of methanogen form a symbiosis by attaching themselves onto protozoa surface (Santoso and Hariadi, 2007). The decreasing protozoa population in rumen is expected to stimulate the decrease of methane gas (CH_4).

Susanti and Marhaeniyanto (2014) claimed that 1% moringa leaves extract reduced methane gas production as well as dry matter and organic matter digestibility. Accordingly, further investigation of <1% Moringa oleifera extract is crucial to reduce methane gas (CH_4) production in sheep. Methane gas production is a waste of wasted feed energy, the higher the methane gas formed, the more detrimental it is because of the lower feed energy efficiency. Therefore, this study examined the administration of *Moringa oleifera* leaf extract to a level of 0.5% in sheep ration on methane gas production, protozoa population, total bacteria, $N\text{-}NH_3$ ammona concentration, and microbial protein synthesis.

Materials and Methods

This research was conducted in a Completely Randomized Design, One Way Classification. The treatments include Moringa leaves (*Moringa oleifera*) obtained from the garden around the campus at 0, 0.25 and 0.50% DM Basis with six replicates (18 treatment units). *In vitro* incubation was performed for four hours according to Sutardi's (1979) method. The materials include fresh ruminal fluid from three newly slaughtered local sheep obtained from an abattoir in Banyumas, Central Java. Moringa leaves were oven-dried at 60ºC for 2 x 24h, ground to powder, and extracted using ethanol. The ration was composed of 60:40 concentrate and ammoniated rice straw (DM basis) (3% added urea). The concentrate included 2:1 rice bran and coconut meal. Ration nutrient content is presented in Table 1. The variables measured include total protozoa, methane gas production, total bacteria, N-NH3 concentration and

microbial protein synthesis in the rumen. Data were subjected to analysis of variance, followed by Orthogonal Polynomial Test (Steel and Torrie, 1993).

Three treatments under investigation:

P1 = Concentrate (60%) + ammoniated rice straw (40%) + 0,00% *Moringa oleifera* leaves extract

P2 = Concentrate (60%) + ammoniated rice straw (40%) + 0,25% *Moringa oleifera* leaves extract

P3 = Concentrate (60%) + ammoniated rice straw (40%) + 0,50% *Moringa oleifera* leaves extract

The variables measured include total protozoa, methane gas production, total bacteria, N-NH_3 concentration and microbial protein synthesis in the rumen. Data were subjected to analysis of variance, followed by Orthogonal Polynomial Test (Steel and Torrie, 1993)

Procedure:

Preparing Moringa oleifera leaves extract

Moringa oleifera leaves were obtained from the neighbourhood of Animal Science Faculty, Jenderal Soedirman University. The leaves were oven-dried at 60 ^{o}C for 2x24 h and ground to powder using a blender. As much as 300 g *Moringa oleifera* leaves meals was dissolved in 2.25 L 96% ethanol, soaked for 2x24h, and stirred in a *Stirer*.

The solution was paper-filtered, and the supernatant was solidified using a *vacum rotary evaporator*. The solid extract was air-dried to eliminate the residual ethanol. The ethanol extract was mixed with concentrate binder with a ratio of 1:1, oven-dried at 60 ^{o}C and ground in a blender for further analysis.

In vitro experiment

The in vitro experiment was conducted using Sutardi's (1979) method. Measuring Total Protozoa.

Methyl green Formalin Salin (MFS) solution was made by mixing and diluting 20 ml formaldehid (35%) with 180 ml aquades, 0.12 g methylgreen, 1.6 g NaCl.

Procedure:

1. The ruminal fluid across treatments was incubated for 4h, then filtered and homogenized.
2. ml filtered ruminal fluid was taken and homogenized, and added with 0.1 ml MFS solution + 0.3 ml aquades.
3. Total protozoa were calculated using a microscope, or the sample stored in a freezer.
4. Total protozoa = N/5 x 10^4 x 5
   Note: 5 is the total compartments and dilution. (Suryahadi, 1990 and Dehority, 1993.

Table 1. The Proportion of Ingredients and Nutrient Composition

Ingredients (%)	Treatments		
	Control	Extract Moringa 0.25%	Extract Moringa 0.50%
Rice straw ammoniated	40	40	40
Rice bran	40	40	40
Coconut meal	20	20	20
Total	100	100	100
Extra Moringa Leaves	0	0.25	0.25
Nutrient Composition			
Crude Protein	10.6	10.6	10.6
Total Digestible Nutrient	50.0	50.0	50.0
Extract Ether	4.2	4.2	4.2
Crude Fiber	27.1	27.1	27.1

Note: Analysis result in Laboratory of Animal Nutrition and Feed Science, Faculty of Animal Science, Jenderal Soedirman University (2018)

Measuring methane gas production

Methane gas production was measured using the concentration data from three main components of volatile acids, i.e., acetic acid, butyric acid, and propionic acid, using a formula by Tamminga (1982): CH_4 methane = 0.5 [acetic] + 0.5 [butyric] – 0.25 [propionic]

[acetic]: acetic acid concentration

[butyric]: butyric acid concentration

[propionic]: propionic acid concentration

Measuring N-NH_3 concentration

N-NH_3 concentration was measured using a Conway's microdiffusion technique (Department of Dairy Science. 1966).

Measuring Microbial Protein Synthesis in Ruminal Fluid

Microbial protein synthesis was measured using a method by Zinn and Owens (1995)

Results and Discussion

Total Protozoa

Protozoa in rumen often disturb bacteria ecosystem because protozoa prey on bacteria and negatively affected fiber digestion. Therefore, it is important to conduct protozoa defaunation. One defaunation agent to limit protozoa population is saponin – the product of the plant's secondary metabolism (Masruroh et al. 2013).

The study on *Moringa olifera* leaves extract showed that the most total protozoa (63.00 ± 9.00 x 10^4 cell/ml) was identified in ruminal fluid given control feed (without *Moringa oleifera* leaves extract) and the lowest was in ruminal fluid with 0.50% *Moringa oleifera* leaves extract (Table 2), which reduced total protozoa up to 95.24%.

The analysis of variance result showed that *Moringa oleifera* leaves extract had a profoundly significant affect (P<0.01) on the total protozoa of ruminal fluid. The Orthogonal Polynomial test indicated a linear effect with an equation of Y = 57.806 – 119.67X, with coefficient determination (r^2) 0.88. (Figure 1). The equation illustrates that the higher level of *Moringa oleifera* leaves extract, the lower total protozoa. It is because *Moringa oleifera* leaves contain saponin, the defaunation agent. (Thalib 2008). Saponin is the secondary group of plant compound that could modify ruminal fermentation and improve cattle production (Wallace et al. 2002).

Saponin extracted with methanol from guar meal was reported to hemolyze red blood cells and inhibitted the activities of pathogenic bacteria, such as *Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* (Hasan et al. 2010). Saponin's mechanism to eliminate protozoa is similar to that of red blood cell haemolysis. Saponin binds cholesterol from the outer membrane of protozoa which punctures a hole and breaks protozoa's membrane (Wallace et al. 2002). The other mechanism is saponin, like detergent, can lower the surface pressure, which renders toxic to protozoa (Franzis et al. 2002). The degradation rate [^{14}C] of leucine from *Selenomonas ruminantium* shows the diminishing activity of bacteria-eating protozoa.

Most *in vitro* experiment showed that saponin activity diminished protozoa population in a rapid and non-selective manner. Koenig et al. (2007) reported that protozoa population in sheep's rumen had significantly decreased within two hours after being fed with saponin-fortified *Enterolobium cyclocarpum*. The population of all protozoa species diminished due to saponin, both in vitro and in vivo. Accordingly, the higher *Moringa oleifera* leaves extract in feed, the lower the total protozoa.

Methane gas production

Methane gas from ruminant cattle has negatively affected the environment and cattle. The methane gas produced in the rumen reflects the loss of energy from the consumed feed; it also indicates low feed efficiency (Tapio et al., 2013). This study indicated that the higher methane gas production was obtained from

ruminal fluid not supplemented with Moringa oleifera leaves extract, and the lowest was in ruminal fluid plus 0.5% Moringa oleifera leaves extract (Table 2). Supplementing Moringa oleifera leaves extract up to 0.50% could reduce methane gas production up to 44.9%.

Analysis of variance showed that supplementing Moringa oleifera leaves extract highly significantly affected (P<0.01) methane gas production. The result of the orthogonal polynomial test showed that the effect was linear with an equation of Y = 34-31.817X, and the coefficient determination (r^2) was 0.78 (Figure 2).

Figure 2 illustrates that the higher level of *Moringa oleifera* leaves extract, the lower the methane gas production. According to Sutton et *al*. (2003) methane gas production was closely related to total acetic acid and butyric acid produced during ruminal fermentation; however, it did not correlate with propionic acid production. This study confirmed the findings that the higher the acetic concentrate, the higher the methane gas production (Figure 3). The correlation was expressed in an equation of Y = 2.24 + 0.874X, and the coefficient determination was (r^2) 0.98. The correlation is strong as observed from the coefficient determination value which illustrates that 98% of methane gas produced is affected by acetic acid. It is because the methane gas produced was dependent on H_2 and CO_2 supply in the rumen, which was released during the formation of acetic and butyric acids in ruminal feed fermentation. In contrast, propionic acid production is not followed by H_2 and CO_2 (Sutton et al., 2003). This study (Table 2) showed that the higher level of *Moringa oleifera* leaves extract, the lower acetic and butyric acids production. Therefore, H_2 and CO_2 supply released during the acid production also diminished which resulted in the decreasing yield of methane gas. The formation of methane gas is carried out by this methanogenic bacterium.

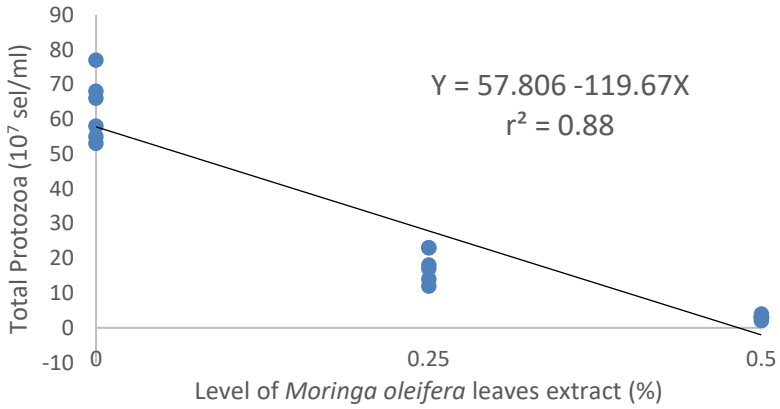

Figure 1. The effect of *Moringa oleifera* leaves extract on Total Protozoa (10^4 cell/ml)

Table 2. The effect of supplementing Moringa oleifera leaves extract on ruminal fermentation products

Ruminal Fermentation Products	Treatments		
	Control	Extract Moringa 0.25%	Extract Moringa 0.50%
Total protozoa (10^4 cell/ml)	63.00 ± 9.00	18.00 ± 5.00	3.00 ± 1.00
Methane gas production (mM)	35.41 ± 4.40	23.24 ± 2.42	19.50 ± 1.56
Total bacteria (10^7 cfu/ml)	4.99 ± 1.19	5.37 ± 0.66	6.19 ± 0.49
N-NH_3 concentration	12.00 ± 0.00	12.92 ± 0.49	13.83 ± 0.41
Microbila protein synthesis (mg/ml)	488.17 ± 7.53	554.83 ± 12.11	679.83 ± 33.27

There are two methanogen bacteria: hydrogenotrophic which converts hydrogen and carbon dioxide to methane, and acetotrophic which converts acetic acid to methane and CO_2 (Mackie and Bryant, 1984). Also, the decreasing methane gas production may due to the increasing level of *Moringa oleifera* leaves extract. It is because the bacteria that produce methane gas form a symbiosis with protozoa. Hidayah (2016) stated that protozoa are the host of methanogen, while Newbold et al., (2015) mentioned that some methanogen live in the rumen by attaching themselves onto protozoa cell membrane. Around 20% (Bryden and Annison, 1998) to 37% (Newbold et al. 1995) methanogen form a symbiosis with protozoa, while the other 63-80% are freely living in the rumen.

Saponin can inhibit the growing population of protozoa because saponin can bind a sterol on protozoa membrane cells and affect the surface pressure of the membrane. As a result, the membrane cells break, the cells undergo lysis and, eventually, the protozoa and the bacteria producing methane gas die (Masruroh et al. 2013),

Total Bacteria (Total Plate Count)

Bacteria population in the rumen constitutes 10^8–10^{11}/g ruminal content. Bacteria is the main source of protein for ruminant cattle. The total and types of bacteria are dependent on the types of feed in the rumen. The study on *Moringa oleifera* leaves extract for feed indicated that the lowest bacteria population (4.99 ± 1.19 x 10^7 cfu's/ml) was observed from ruminal fluid without *Moringa oleifera* leaves extract, and the highest population (6.19 ± 0.49 x 10^7 cfu's/ml) was in ruminal fluid plus 0.50% (DM basis) *Moringa oleifera* leaves extract (Table 2). Supplementing *Moringa oleifera* leaves extract up to 0.50% increased total bacteria up to 24%.

The analysis of variance result showed that supplementing *Moringa oleifera* leaves extract significantly affected (P<0.5) total bacteria. The orthogonal polynomial test indicated a linear effect with an equation of Y = 4.7147 + 2.6233X, with coefficient determination (r^2) of 0.32. Although the effect of *Moringa oleifera* leaves extract on total bacteria was only 32%, Figure 4 illustrates that the higher level of *Moringa oleifera* leaves extract, the higher the total bacteria (total plate count). It is because *Moringa oleifera* leaves extract could reduce total protozoa which feed on bacteria. The increasing total bacteria was linear to the supplementation of *Moringa oleifera* leaves extract because *Moringa oleifera* leaves contained high protein, i.e., 27.10 g protein/100 g dry leaves, which mainly supplies nutrients for the rumen microbes, particularly bacteria.

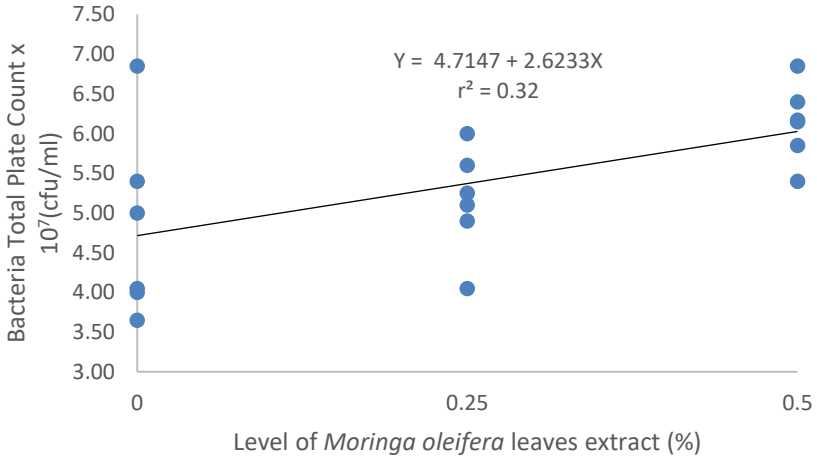

Figure 4. The effect of *Moringa oleifera* leaves extract on Bacteria Total Plate Count (10^7 cfu/ml)

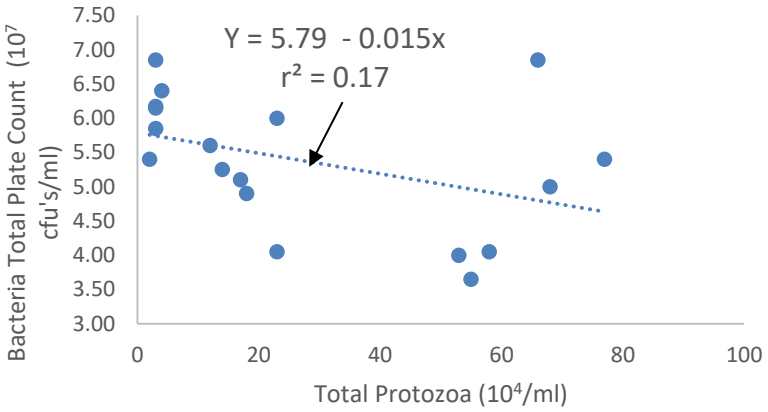

Figure 5. Relationship between Total Protozoa and Total Bacteria in Ruminal fluid Supplemented with *Moringa oleifera* leaves extract

There was a correlation between total protozoa and bacteria – the higher the total protozoa in the ruminal fluid, the lower the total bacteria, and vice versa (Figure 5). In the rumen, protozoa are beneficial for ruminal ecosystem but also detrimental as they devour bacteria (low-quality feed). Despite the low coefficient determination (0.17), Figure 5 illustrates that as the total protozoa increased, total ruminal bacteria decreased.

N-NH_3 ammonia concentration

Concentration of ammonia-N ruminal fluid was measured to observe the activity of ruminal microorganism in protein degradation. NH_3 in the rumen is dependent on protein and amino acid content. Ammonia is the product of amino acid deamination by microbial activity, so the concentration is affected by the digestible protein content in the rumen (Batch et al., 2005). In rumen, protein undergoes hydrolysis and is converted to peptide by the activity of microbial enzymes. Half of peptide is used to form protein in microbial cells and amino acids. The amino acid is deaminated into NH_3 by microbial activity; therefore, the NH3 in the rumen is dependent on protein feed content (Pamungkas, 2008).

The average N-NH_3 concentration in this study was 12.00 ± 0.001 to 13.83 ± 0.41 (Table 2). Supplementing *Moringa oleifera* leaves extract up to 0.50% increased N-NH_3 by 15.25%. The average N-NH_3 in this study (Table 2) was within the normal range for microbe for an optimum feed digestibility. According to Paengkoum et al. (2006) N-NH_3 concentration for microbes to perform an optimum feed digestibility was 5-20 mg/ dL, or equal to 3.57-14.28 mM. Accordingly, the average N-NH_3 in this study was within the normal range, but higher than Hendratiningrum et al. (2011), i.e. 6.67-7.82 mM. The gap was due to the contributing factors of N-NH_3 production such as feed, particularly the composition and protein content (Suwandyastuti and Rimbawanto, 2011). The analysis of variance result showed that supplementing *Moringa oleifera* leaves extract had a highly significant effect ($P<0.01$) on N-NH_3 concentration. The Orthogonal polynomial test indicated that the higher level of *Moringa oleifera* leaves extract may increase N-NH_3 concentration, with an equation of $Y = 12 + 3.67X$, with a coefficient determination (r^2) of 0.83 (Figure 6). The equation illustrates that when the level of *Moringa oleifera* leaves extract increased by one unit, the concentration of N-NH_3 would increase 3.67 unit and N-NH_3 ruminal fluid in this study was affected by *Moringa oleifera* leaves extract (83%).

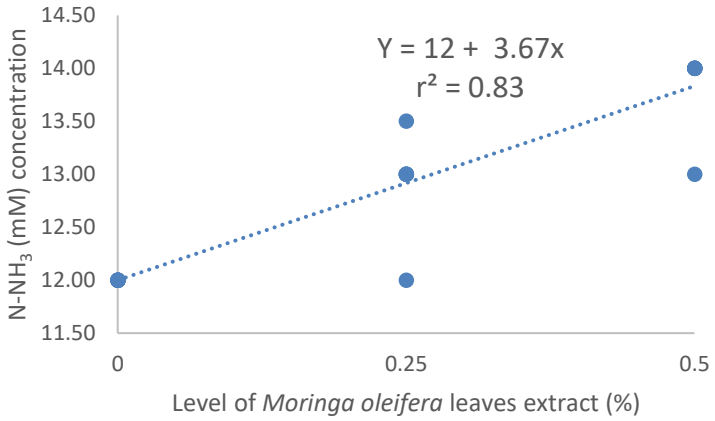

Figure 6. The effect of *Moringa oleifera* leaves extract on N-NH_3 concentration

The analysis of variance result showed that supplementing *Moringa oleifera* leaves extract had a highly significant effect (P<0.01) on N-NH_3 concentration. The Orthogonal polynomial test indicated that the higher level of *Moringa oleifera* leaves extract may increase N-NH_3 concentration, with an equation of Y = 12 + 3.67X, with a coefficient determination (r^2) of 0.83 (Figure 6). The equation illustrates that when the level of *Moringa oleifera* leaves extract increased by one unit, the concentration of N-NH_3 would increase 3.67 unit and N-NH_3 ruminal fluid in this study was affected by *Moringa oleifera* leaves extract (83%).

The increase of N-NH_3 concentration linear to *Moringa oleifera* leaves extract supplementation was due to the high protein content in moringa leaves (24.02%) and protein fermentation produced N-NH_3. Therefore, supplementing Moringa leaves extract in feed would improve protein supply for ruminal fluid, and eventually, N-NH_3 would increase.

Microbial protein synthesis in the rumen

The advantage of ruminants over other cattle is the biological process by rumen microbes can convert fibrous feed, low-quality protein feed even non-protein nitrogen to beneficial nutrition for the ruminants. Two-thirds to three-fourths of amino acid absorbed by ruminants are derived from microbial protein in the rumen. Therefore, the rumen microbes are an important source of protein for ruminants.

This study reported that the average microbial protein synthesis in the rumen ranged from 488.17 ± 7.53 mg/ml to 679.83 ± 33.27 mg/ml (Table 2). Supplementing 0.50% Moringa oleifera leaves extract improved microbial protein synthesis in the rumen by 39.26%. This average was higher than that of Syamsi et al (2018), i.e., 103.67-142.55 mg/20 ml. The gap was because microbial protein synthesis in the rumen is strongly dependent on the availability of N-NH3 precursor (the N source to compose microbial protein) and the availability of energy derived from fermentation (as the source of energy and carbon skeleton) (Suryapratama and Suhartati, 2012). N-NH3 is the product of protein fermentation in the rumen. Supplementing 0.50% Moringa oleifera leaves extract increase protein supply for the rumen microbes, which increased fermentation product (N-NH3) and improve microbial protein synthesis in the rumen.

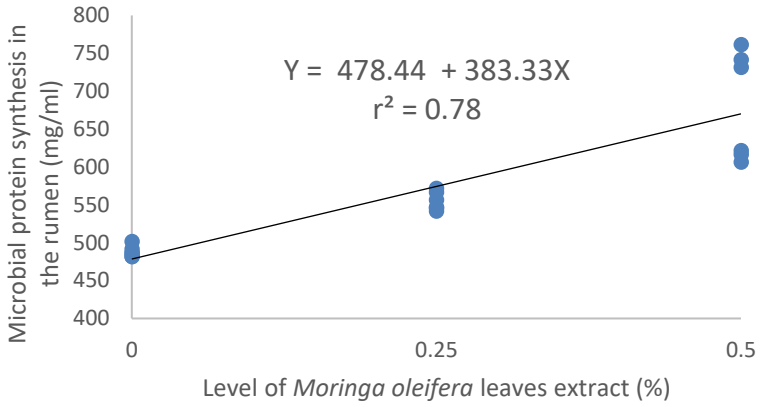

Figure 7. The effect of *Moringa oleifera* leaves extract on Microbial Protein Synthesis in the rumen

Supplementing *Moringa oleifera* leaves extract was highly significant to microbial protein synthesis in the rumen ($P<0.01$). Orthogonal Polynomial test illustrates a linear response with an equation of Y = 478.44 + 383.33X and the coefficient determination (r^2) was 0.78 (Figure 7). It indicates that the higher the level of *Moringa oleifera* leaves extract, the higher the microbial protein synthesis in the rumen. The improvement was indicative of an increasing N-NH_3 supply due to a higher level of *Moringa oleifera* leaves extract. As mentioned before, microbial protein synthesis in the rumen was very dependent on N-NH_3 production. Figure 6 illustrates that the higher the *Moringa oleifera* leaves extract, the higher the N-NH_3 concentrate. Ginting (2005) explained that the main supplier of ammino chain for microbial protein synthesis is ammonia derived from protein degradation, NPN degradation in feed, or the recycled urea degradation. Therefore, the higher level of *Moringa oleifera* leaves extract would increase N-NH_3 concentration and, eventually, microbial protein synthesis in the rumen.

Conclusions

Ruminal fluid supplemented with 0.50% *Moringa oleifera* leaves extract (DM basis) can suppress total protozoa and methane gas production in the rumen. Supplementing *Moringa oleifera* leaves extract 0.50% could lower total protozoa by 95.24%, and methane gas production by 44.9%, as well as increasing total bacteria, N-NH_3 concentration and microbial protein synthesis by 24%, 15.25% and 39.26%, respectively.

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