Effect of *Leucaena leucocephala* substitution on *in vitro* rumen fermentation and methane emission in thin tailed-sheep

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**Abstract.** This study aimed to evaluate the effects of substitution of *Leucaena leucocephala* (LL) leaves on *in vitro* rumen fermentation and methane emission in thin tailed-sheep. Rumen fluid were collected from two thin tailed-sheep fed with mixed-forages. The basal diet used in *in vitro* study consisted of 30% of concentrate and 70% of forages. LL leaves substituted the forages of basal diet by 10% or 25%. Methane production, carbon dioxide (CO₂), ammonia concentration, microbial protein, protozoa, volatile fatty acids (VFA) were obtained according to *in vitro* gas production methods followed by microbial diversity evaluation. Substitution of LL leaves reduced (P<0.05) the pH, ammonia and microbial protein regardless percentage of substitution. Protozoa counts lowered as the LL leaves increased (P<0.05). However, there were no significant effects of LL leaves substitution on VFA production, CO₂ and methane emission. It can be concluded that substitution of LL leaves to forages up to 25% was not enough to reduce methane emission although some ruminal fermentation parameters were affected.

1. Introduction

Climate change is global issue that drives the changes in ecological aspects, as well as social, economics, politics and many others. Methane (CH₄) is one of the main greenhouse gas (GHG), together with carbon dioxide (CO₂) and nitrous oxide (N₂O) [1], that play important role in climate change. Pastoral lands particularly for ruminants grazing is often to be associated as the main factor for GHG emission since CH₄ emission from herbivore is 24.5 time more powerful than CO₂ [2]. On the last few years, research on strategies to mitigate enteric CH₂ production by ruminants have been conducted [3,4]. Mitigation through nutritional strategies is being the most developed and reported in detailed and critical way than biotechnologies and additives strategies [2]. However, most attempts to mitigate CH₂ production through varying dietary forages resulted in a reduction in ruminal fiber degradability or total-tract nutrient digestibility [5–6].

There is growing interest in the use of plant secondary compounds for CH₄ mitigation, and tannins is one of those which is most reported to have positive effect, beside saponin. The anti-methanogenic activity in tannin-containing plants has been attributed mainly to the group of condensed tannins, which decreases the protozoa population, methanogenic archaea [7–8], and the synthesis of enteric CH₂ [8–9]. *Leucaena leucocephala* (LL) is one of the main Indonesian legumes which contains CT with methane reducing potential. Others reported that LL improve CP digestibility, N retention and improve the efficiency of energy intake by ruminants [9–10]. This research aims to add information...
regarding the LL effects on rumen fermentation, methane emission and microbial diversity particularly in thin-tailed sheep.

2. Material and methods

2.1. Materials
Rumen fluid were obtained from two male thin-tailed sheep (±2 years old; body weight ±25 kg). Basal diet consisted of 70% of king grass and 30% of concentrate mixture (85% soy bean meal and 15% wheat pollard). LL was obtained in Gunung Kidul area. Fresh LL was aerated-drying for 3 d prior to oven-drying for 7 d.

2.2. Methods
Prior to slaughter, sheep were fed with basal diet (ad libitum) twice per day for 10 d. Sheep were slaughtered in the morning on the next day and ruminal fluid was collected and prepared prior to in vitro study. Feed samples for in vitro were basal diet (BD), or 10% (w/w) or 25% (w/w) LL substitution to king grass in BD. In vitro ruminal gas fermentation was conducted in triplicates. Feed samples, buffers and rumen fluid were incubated for 24 h at 39°C. The pH, ammonia, microbial protein, protozoon population, volatile fatty acid (VFA) and methane and CO2 emission were measured after incubation.

Data were analyzed with one-way ANOVA followed by Duncan’s multiple range test (DMRT) in SPSS 2016.

3. Results and discussion
Twenty five percent (25%) substitution of king grass with LL significantly lowered the pH and decrease ammonia production (P<0.05), but 10% substitution had no effect on those parameters (Table 1). Although 25% LL lower the pH, the value was in the normal range of rumen pH, which is 6.3 to 7.0 [1]. The lower ammonia obtained from 25% LL substitution might be due to the tannin content in LL which act as protein binder and protected protein from rumen degradation, resulted in a lower ammonia production [11]. The LL used in this study contain 5.5% total tannin and 0.45% condensed tannin (DM based; data not shown).

Table 1. Effects of substitution of Leucaena leucocephala (LL) leaves on in vitro rumen fermentation in thin tailed-sheep

| Parameters            | BD*      | 10% LL       | 25% LL       |
|-----------------------|----------|--------------|--------------|
| pH                    | 6.98 ± 0.01<sup>b</sup> | 6.97 ± 0.003<sup>ab</sup> | 6.96 ± 0.003<sup>a</sup> |
| NH3 (mg/100 ml)       | 76.36 ± 3.23<sup>b</sup> | 62.74 ± 4.40<sup>ab</sup> | 57.73 ± 5.23<sup>a</sup> |
| Microbial protein     | 0.22 ± 0.01<sup>b</sup> | 0.17 ± 0.01<sup>a</sup> | 0.16 ± 0.01<sup>a</sup> |
| Protozoa (10<sup>3</sup> cell/ml) | 78.41 ± 0.28<sup>c</sup> | 58.08 ± 0.07<sup>b</sup> | 30.58 ± 0.43<sup>a</sup> |
| VFA                   |          |              |              |
| Acetate               | 31.56±1.36 | 35.66±2.79 | 33.03±0.20  |
| Propionate            | 12.33±1.42 | 12.15±1.17  | 10.85±0.72  |
| Butyrate              | 5.15 ± 0.56 | 5.38 ± 0.60 | 4.53 ± 0.35 |
| Total VFA             | 49.04±3.34 | 53.19±4.56 | 48.41±1.27  |
| Acetate:Propionate    | 2.61 ± 0.24 | 2.95 ± 0.11 | 3.07 ± 0.21 |

<sup>a, b, c</sup> different superscript on the same row expressed statistically difference at (P<0.05); *BD = Basal Diet, 70% of king grass and 30% of concentrate mixture (85% of soy bean meal and 15% of wheat pollard); 10% LL and 25% LL= 10% (w/w) or 25% (w/w) LL substitution to king grass in BD.

King grass substitution with 10% or 25% LL significantly decreased protozoa counts and microbial protein (P<0.05) but they had no effect on VFA production. Again, the antiprotozoal effect of LL might be attributed to high tannin contained in LL leaves. Similar findings were reported by other
studies, in which the condensed tannins of tropical trees or legumes have the capacity of reducing rumen protozoa and bacteria populations [8]. These findings are in accordance with the microbial protein results that the lower protozoa and bacteria population the reduced microbial protein synthesis.

Min et al. [8] reported that condensed tannins reduce the protozoa and methanogenic bacteria population and it is linked to the reduction of methane products in rumen. It is also reported that protozoa are responsible for 37% of CH4 emissions related to their role in the fibrous fraction of feedstuffs degradation up to 50% [12–13]. Although the reducing effect of 10% or 25% LL substitution on protozoa and bacteria population are also reported in our study, the VFA, methane and CO2 emission were not affected. It might be related to the low percentage of LL substitution. Addition of LL at 30% [14] and ranged from 30-60% [15] significantly reduced in total and ciliate protozoa and methane emission.

Table 2. Effects of substitution of *Leucaena leucocephala* (LL) leaves on methane and CO2 emission

|                  | BD* | 10% LL | 25% LL |
|------------------|-----|--------|--------|
| Methane (ml)     | 1.26 ± 0.21 | 9.48 ± 1.89 | 9.43 ± 0.89 |
| Methane (ml)/DDM | 0.08 ± 0.01 | 0.06 ± 0.01 | 0.08 ± 0.01 |
| Methane (ml)/DOM | 0.11 ± 0.01 | 0.12 ± 0.01 | 0.13 ± 0.02 |
| CO2 (ml)         | 41.25 ± 1.99 | 36.67 ± 4.87 | 35.18 ± 3.39 |

*BD = Basal Diet, 70% of king grass and 30% of concentrate mixture (85% of soy bean meal and 15% of wheat pollard); 10% LL and 25% LL= 10% (w/w) or 25% (w/w) LL substitution to king grass in BD.

4. Conclusion
Substitution of LL leaves to forages basal diet up to 25% was not enough to reduce methane emission although some ruminal fermentation parameters were affected.

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