Methane Production and Methanogens Diversity in *in vitro* Ruminal Fermentation with Mahogany Leaves Meal (*Swietenia mahagoni*) as Tannin Source

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Abstract. *Swietenia mahagoni* has been extensively studied and applied as feed additives, a strategy to manipulate rumen fermentation in reducing methanogenesis. The research aimed to determine the effect of using *Swietenia mahagoni* as tannin source on methane production, gas production kinetics and the diversity of methanogenic archaea by *in vitro* rumen fermentation. *Swietenia mahagoni* was given to substitute the elephant grass at levels of 0, 25, and 50%. Fermentation was carried out by *in vitro* gas production technique according to Menke and Steingass. Methane was analyzed using gas chromatography (GC) method. Terminal restriction fragment length polymorphism (TRFLP) analysis was used to detect changes of rumen methanogenic archaea diversity. Result showed that *Swietenia mahagoni* leaves with level 50% reduced methane production by 39.26% (P=0,08). Gas production from insoluble fraction (b) decreased as much as 47.23% from control with using *Swietenia mahagoni* leaves 50%. The uncultured *methanococcales* was dominating community member 36.35% with *Msp*l, while uncultured euryarchaeote clone is 80.24% MCR-F1SP-1 with *Taq*l. Inclusion of *Swietenia mahagoni* as much as 50% in diet could decrease methane production, gas production from potential degraded fractions and diversity of methanogenic archaea.

1. Introduction

The ruminants animal rely on rumen microbes to convert feed components into useable source of energy and protein. Rumen microbes ferment feed particle and produce volatile fatty acids (VFA) and gases such as methane. Methane is the undesired by-product of feed fermentation process in the rumen. Methane is known to cause global warming, also shows the loss of energy that should be utilized by livestock [1]. Various efforts were developed to reduce methane gas emissions [2]. Mahogany (*Swietenia mahagoni*) is reported to be used as an additive to reduce methane gas production. This is because mahogany contains secondary metabolites in the form of tannins [1]. The tannin content in mahogany is quite high at 11,928 mg / 100 mg [3]. Tannins have also been reported to cause changes in rumen microbial diversity because tannins can bind proteins to both protein feed and microbial protein [4]. Methanogenesis is played by methanogenic bacteria utilizing H₂, CO₂, formate and acetate substrates [5]. Methanogenic archaea are quite difficult to be cultured, so to identify the presence of methanogenic can use the TRFLP method [6]. The stages of identification of methanogenic bacteria begin with DNA isolation [6]. The results of isolation were visualized using electrophoresis [7]. Isolated DNA then used as template for amplification by PCR of a gene sequence encode of key enzyme in methanogenesis pathway [8]. The PCR results were then subjected to digestion using endonuclease
restriction enzymes [9]. Profile of restricted fragment then used to characterize the diversity of methanogens by comparing with PCR and digestion in silico.

2. Materials and methods

2.1 Sample preparation
Elephant grass (Pennisetum purpureum), Swietenia mahagoni leaves, pollard, and soybean meal were dried in an oven at 55°C for 5 days and then ground and stored. The nutritional composition of raw materials measured includes dry matter (DM) and organic matter (OM) analyzed using the proximate method.

2.2 In vitro gas production
In vitro gas production were used in this study according to method explained by [10]. Substrate of 300 mg was put into the syringe and incubated for 48 h under anaerobic condition at 39°C. Rumen fluid and buffer mixture (1:2) was pumped in 30 ml doses into syringes. The dietary treatments were: P0 (100% elephant grass + 0% mahoni leaves + 30% concentrates), P1 (75% elephant grass + 25% mahoni leaves + 30% concentrates), and (50% elephant grass + 50% mahoni leaves + 30% concentrates). Each treatments was examined in triplicate. At the end of incubations gas samples were collected using calibrated syringe and 10 ml of gas were transferred into 10 ml plain vacuum tube for methane determination using gas chromatography. Gas production kinetics, its determination using FitCurve programme [11].

2.3 T-RFLP analysis
The diversity of methanogenic bacteria was determined using terminal restriction length polymorphism (T-RFLP) [2]. DNA samples were extracted following the protocol of ZymoBIOMICSTM DNA mini kit catalog No. D4300.

The gene of methyl-coenzyme M reductase –subunit A (mcrA) was amplified using primers specific for mcrA MLf (5′-GGT GGT GTM GGA TTC A CAR TAY GCW ACA GC-3′) and MLr (5′-TTC ATT GCR TAG TTW GGR TAG TT-3′) [12]. The forward primer for the PCR reaction was labeled on the 5′ end with 6-fluorescein amidite (FAM) marker to allow the terminal fragment to be tracked.

Concentration of DNA for PCR template was adjusted about 50 μg/μl by dilution. The PCR reaction mixture contained 12.5 μl GoTaq® Green Master Mix (Promega Corporation), 1 μl each forward and reverse primers, 1 μl DNA template, and 9.5 μl DNase free water. The PCR conditions were pre-denatured 95 ° C for 5 minutes, 35 denaturation cycles of 95 °C for 1 minute, annealing 55 ° C for 45 seconds and elongation 72 °C for 1 minute, and final extension 72 ° C for 10 minutes. The PCR product was then verified with 1% agarose gel electrophoresis. The next three replication tubes were compiled and cut with TaqI restriction enzymes and MspI Fast Digest (Thermofisher Scientific) at 20 μl volume containing 16 μl pure PCR products, 2 μl 10X Fast Digest® buffer, 2 μl enzymes. Digestion was carried out at 37°C for 5 minutes for MspI, whereas TaqI. at 65°C for 5 minutes. Cutting results were analyzed with AB13100 genetic analysis system (applied biosystem). <50 bp and> 500 bp peaks were not included in the analysis. The TRF pattern was compared with the results of in silico cutting with MICA 3 software.

2.4 Data analysis
Data of fermentation were analyzed using one-way analysis of variance with SPSS 16 for Microsoft Windows and statistically significant differences between means were determined by Duncan’s Multiple Range Test (DMRT) when the effects of treatment (P<0.05) were detected, microbial diversity data were analyzed descriptively [13].
3. Result and discussion

3.1. In vitro methane and gas production kinetics

Methane production and gas production kinetics were presented in Table 1. Tannin from mahogany leaves at levels 25% did not affect significantly on methane production per dry matter diet but decreased methane production in higher levels (50%). The higher the provision of tannin, the lower the methane gas produced [8]. The rate of gas produced (c) from degraded feed was decreased with increased levels of mahogany. Hence, gas production also decrease with increased time spent on incubation [14].

Table 1. Effect of inclusion *Swietenia mahagoni* as tannin source on methane production and gas production kinetics

| Parameters | Level of *Swietenia mahagoni* (%) |
|------------|-----------------------------------|
|            | 0   | 25  | 50  |
| CH₄/DM (ml/g) | 87.43 ± 9.01 | 84.86 ± 6.31 | 53.15 ± 12.75 |
| a (ml)      | 2.60 ± 0.17 | 3.14 ± 0.22 | 3.51 ± 0.32 |
| b (ml)      | 65.43 ± 0.43<sub>y</sub> | 55.73 ± 2.56<sub>x,y</sub> | 34.53 ± 11.82<sub>x</sub> |
| a+b (ml)    | 68.03 ± 0.60 | 58.87 ± 2.69 | 38.03 ± 12.13 |
| c (ml/jam)  | 0.06 ± 0.00 | 0.06 ± 0.00 | 0.11 ± 0.01 |

<sup>ns</sup> Non significant.
<sup>x,y,z</sup> Means with different superscripts in the same row differ at P<0.05

3.2 T-RFLP profile of methanogenic community

The DNA band as result of PCR amplification were produced a single band of DNA that measure approximately 500 bp at 1% agarose of electrophoresis (Figure 1).

![Figure 1. DNA band as result of methanogen mcrA gene by PCR amplification. (1, 2, 3: inclusion of 0 (control), 25 and 50% *Swietenia mahagoni* leaf in the diet.](image)

The same primer also used by [12] which showed that the amplification product has a fragment length between 464 to 491 bp. A total of 6 TRF’s were identified as methanogen from digestion product with MspI and 4 TRF’s from digestion with TaqI restriction enzyme (Table 2). The abundance of methanogenic bacteria based on (Figure 2) shows the change in diversity at the level of mahogany leaf 0, 25 and 50%. Histogram of relative abundance is constructed based on TRF data from MspI and TaqI digestion which had been analyzed using MICA 3.
Table 2. Terminated restriction fragment size of amplicon mcrA gene digested with restriction enzyme MspI and TaqI

| Restriction Enzyme | No  | Organism                                      | Size of DNA Fragment (bp) |
|--------------------|-----|-----------------------------------------------|---------------------------|
| MspI               | 1.  | Uncultured archaeon clone ATB-EN10710         | 127                       |
|                    | 2.  | Methanococcus maripaludis C5"                 | 183                       |
|                    | 3.  | Uncultured Methanobrevibacter sp.             | 193                       |
|                    | 4.  | Uncultured methanogenic archaeon clone P23-1  | 252                       |
|                    | 5.  | Uncultured Methanoculleus sp.                 | 255                       |
|                    | 6.  | Methanotorris igneus Kol 5"                   | 469                       |
| TaqI               | 1.  | Uncultured archaeon clone ATB-EN10715         | 50                        |
|                    | 2.  | Uncultured archaeon clone CLIO1               | 201                       |
|                    | 3.  | Uncultured archaeon clone CLI36               | 204                       |
|                    | 4.  | Uncultured euryarchaeote clone MCR-F1SP-1     | 432                       |

In this present, digestion of amplicon products using MspI restriction enzyme resulted TRFs with sizes from 127 to 469 bp, whereas for TaqI restriction enzyme between 50 to 432 bp. TFRs identification from MspI digestion product shows that Uncultured Methanobrevibacter sp. only observed in treatment 0%. There is different with Methanococcus maripaludis CS only observed in treatment 50%. Data TRF from TaqI digestion shows that Uncultured archaeon clone CLI36 and CLIO1 only observed in treatment 0%.

![Abundance of methanogenic bacteria based on TRFs analysis of mcrA gene digest with MspI (a) and TaqI (b) restriction enzymes](image)

**Figure 2.** Abundance methanogenic bacteria based on TRFs analysis of mcrA gene digest with MspI (a) and TaqI (b) restriction enzymes

Abundance of methanogenic bacteria based on Figure 2 shows that TFR produced from digestion with MspI and TaqI was changed indicating the changing of diversity of microbes. Our results is in accordance with [15] who reported about the changes of rumen microbes due to tannin effect.
4. Conclusion

Inclusion of *Swietenia mahagoni* leaves at level 50% did not significantly affect the production of methane and the kinetics of gas production. The digestion with *MspI* and *TaqI* restriction enzyme resulted six and four TRF from all samples, respectively.

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