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In vitro gas production kinetics and digestibility of king grass (Pennisetum hybrid) added by organic mineral and natural crude tannin

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**ABSTRACT**

This research was conducted to compare kinetics of gas production, methane emission, and in vitro digestibility between organic mineral (OM) and inorganic mineral (IM) in king grass (Pennisetum hybrid), in combination with natural crude tannin from neem (Azadirachta indica, AI) leaves. Treatments were as follows: T0 (king grass as a control), T1 (T0 + 3% IM), T2 (T0 + 3% OM), T3 (T0 + 2% AI), T4 (T0 + 3% IM + 2% AI), T5 (T0 + 3% OM + 2% AI), and T6 (T0 + 40 ppm monensin), and these were arranged on a completely randomized design. Data were analysed using ANOVA and orthogonal contrast test was used for comparing among treatment means. Results showed that either OM or IM supplementation significantly increased \( P < .05 \) gas production. Total gas productions from T0, T3, T5, and T6 were lower than those of T1, T2, and T4. Total VFA, acetate, and propionate were similar in all treatments; however butyrate concentration was higher in T2 and T4 than the others. In vitro organic matter digestibility, protozoa cells number, and ammonia and methane concentrations were not influenced \( ( P > .05 ) \) by treatments. In summary, either OM or IM improved fermentability of king grass while their combination with tannin-containing leaves reduced the fermentability without affecting methane production.

1. Introduction

Mineral is among the essential elements that plays an important role in cattle’s physiological processes for growth and health maintenance. Mineral supplementation through feed is not only to prevent deficiency but also to optimize livestock production and health. Mineral is usually supplied to the livestock through mineral mixture in its inorganic form. However, a major disadvantage of using such a supplement is that the mineral is not fully absorbed due to antagonism and anti-nutritional factors (such as tannin, phytic acid, oxalate, etc.) present in the diet (Bhanderi et al. 2010). Organic mineral (OM) (mineral bound to organic compound) that is easily absorbed by the body is one of the important solutions to overcome insufficient availability of minerals found in forage crops or natural grasses to meet the physiological needs of livestock.

For instance, trace metal–amino acid complexes can allegedly mimic the process by which trace elements are absorbed (as metal-peptides) and thus be more available to livestock than inorganic mineral (IM) (Suttle 2010). Neem (Azadirachta indica) is a plant species that is rich in bioactive compounds. It was observed that neem leaf contained flavanoid and tannin (Pandey et al. 2014). Recently, tannin has been used to modify feed utilization by reduction in methane emission and therefore tannin is considered as a natural compound possessing methane-mitigating effect (Jayanegara et al. 2013).

Further, Biswas et al. (2002) reviewed that many bioactive compounds in neem leaf were cyclic-trisulphide and cyclic tetrasulphide which can be used antivirus, antiparasite and support to humoral immunity. Neem leaf had also been reported as an ethnoveterinary medicine for inhibiting larvae of the sheep nose bot fly (Cepeda-Palacios et al. 2014) and preventing bovine strongylosis (Jamra et al. 2015).

It would be of interest to evaluate the effect of OM combined with neem leaf on rumen fermentation parameters including ruminal methane emission. Therefore, the objective of this experiment was aimed to evaluate the effectiveness of OM supplementation (as compared to its inorganic form) combined with neem leaf on fermentability, methane emission, and in vitro digestibility of feed forages.

2. Material and methods

2.1. Forage, tannin, and mineral preparation

A forage sample was prepared by harvesting king grass (Pennisetum hybrid) at 70 days after planting. Forage was chopped and dried at 60°C for 2–3 days (until moisture content reached 12%), then ground and sieved into 2 mm particle size. A drying method using a vacuum oven was employed according to the AOAC Official Method 934.06 (AOAC 1995). Natural crude tannin was prepared by drying neem (Azadirachta

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indica) leaves according to forage preparation as well. OM was prepared by inoculating Saccharomyces cerevisiae ATCC 9763 (50 ml) into cassava flour as substrate (1 kg) which was fortified by micro-minerals. Prior to fermentation processes, inoculum was cultivated in malt extract broth (Oxoid) and incubated for 24 h at 30°C, similar to the experiment conducted by van Rijswijck et al. (2015). Micro-minerals formulated per kg substrate contained FeCl2.4H2O (0.177 g), MnCl2.4H2O (7.129 g), CuSO4.5H2O (9.810 g), ZnSO4.7H2O (12.646 g), CoCl2.6H2O (0.192 g), and KI (0.217 g). Fermentation was conducted for seven days on the substrate in the facultative condition then dried by oven at 55°C (for up to 24–48 h, moisture content around 10–12%), followed by grinding and sieving to 1 mm particle size. However, IM was prepared by the addition of micro-minerals’ formula on the substrate without fermentation.

2.2. Experimental design and data analysis

Treatments that were arranged on a completely randomized design consisted of seven treatments, that is, T0 (P. hybrid as a negative control), T1 (T0 + 3% IM), T2 (T0 + 3% OM), T3 (T0 + 2% AI), T4 (T0 + 3% IM + 2% AI), T5 (T0 + 3% OM + 2% AI), and T6 (T0 + 40 ppm monensin as a positive control). Monensin was used as a positive control since the substance has as antiprotozoal agent (Kisidayová et al. 2009) associated with reduction of methane emission (Patra & Saxena 2010). Each treatment was conducted in 3 replications. Variables measured were in vitro organic matter digestibility (IVOMD), gas production kinetics, volatile fatty acids (VFAs), ammonia (NH3), methane (CH4), non-glucogenic ratio (NGR), and protozoal number. Data were analysed by the one-way ANOVA and continued with orthogonal contrast test (Gomez & Gomez 1984) when the treatments showed significantly different P < .05.

2.3. In vitro digestibility and fermentability evaluation

In vitro digestibility and fermentability were conducted by gas production technique (Menke et al. 1979) using a 100 ml glass syringe (Fortune Models, Poulten and Graft GmbH). Sample of forage (380 mg), in- or OM (11.4 mg), natural crude tannin (7.6 mg), or monensin (0.0152 mg) (according to treatments) was placed into a syringe for pre-incubation for 24 h at 39°C. Rumen liquor from a fistulated cattle (10 ml) and buffer solution (20 ml) were inserted into each syringe and incubated with CO2 gas. Seven treatments were randomly allocated to an incubator. Incubation was carried out at 39°C and gas production was observed at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36, and 48 hours after incubation. Gas production kinetics was calculated following the equation \( Gp = B \times [1 - e^{-k(t-L)}] \) (López et al. 1999), where \( Gp \) is the cumulative gas production at time \( t \), \( B \) is the maximum gas production (ml/g DM), \( k \) is the gas production rate (ml/h), \( t \) is the incubation time (h), and \( L \) is the lag time (h). Determination of IVOMD was assayed according to Blümmel et al. (1997). Productions of VFA and NH3 were measured at the end of the incubation. Analysis of VFA was performed by the gas chromatography method according to Friggens et al. (1998) and NH3 analysis using the spectrophotometry method as described by Broderick and Kang (1980). NGR value was calculated according to Zhang and Yang (2012).

2.4. Methane measurement and counting protozoa

The concentration of methane from total gas production was sampled at 18 h. Each sample taken included 10 ml of gas using vacuum syringe then injected into a vacuum tube. Gas samples were analysed using gas chromatography (Shimadzu GC-14) equipped with Proparok Q column (50°C) and a flame ionization detector (150°C) as described by Liu et al. (2011). Protozoa cell was counted using a hemocytometer and colouring with methylene green formalin saline/MFS (Ogimoto & Imai 1981). The MFS solution was composed of 100 ml of 35% formaldehyde solution, 900 g of distilled H2O, 0.6 g of methyl green, and 8 g of NaCl.

3. Results and discussion

Supplementation of micromineral (organic or organic form) combined with tannin from neem leaves (Azadirachta indica) significantly (P < .05) affected cumulative \( Gp \), maximum \( B \), gas production rate \( k \), and butyrate. However, the treatment did not affect the IVOMD, total VFA, lag time \( L \), ammonia, and protozoa (Table 1). Furthermore, methane production from feed combined with tannin seems to be lower than the others and NGR value tends to be higher than control (Figure 1). Supplementation of OM and tannin increased butyrate acid and tended to improved \( P \approx .109 \) total VFA production. However, ammonia and protozoa number were not influenced by treatment. Gas production kinetics from forage-supplemented IM (T1), OM (T2), and IM + tannin (T4) showed higher gas production than control and tannin (T3), OM + tannin (T5), and monensin (T6).

Increasing gas production, VFA, and butyrate of forage supplemented by T2 or T4 indicated that OM presence contributed to support ruminal microbes activity in producing VFA. In contrast, addition of tannin and OM (T5) showed reducing VFA which had an antagonism effect on fermentability. Antagonistic mechanism related to chemical characteristic of tannin to bind organic compound consequences reduction of OM by rumen microbe rumen. Tannin (condensed) has a complex strongly binding with metal ions, carbohydrates, and proteins (Porter 1992). Moreover, supplementation of condensed tannin reduced organic matter digestion in rumen. Lorenz et al. (2014) revealed that protein as organic matter could be precipitated by tannin in rumen. It was indicated by gas production and VFA from substrate supplemented with tannin (T3 and T5) less than without tannin addition.

Furthermore, the binding effect of tannin to inhibit protozoa growth in rumin simulated completion utilization of mineral by ruminal bacteria. Bacteria were supplied enough mineral diet while protozoa were inhibited by supplementation of tannin from neem leaves. Biswas et al. (2002) reported the presence of many kinds of bioactive compounds in A. indica such as azadirachtin, polysaccharide, cyclic-trisulphide/tetrasulphide, and glycoside which have antibacterial, antiviral, and anti-inflammatory properties. In ruminal metabolism, tannin (contained in neem leaves) was able to inhibit methanogenic bacteria and protozoa (Patra & Saxena 2010); as a consequence it improved feed utilization by increasing fermentability. In contrast, Vasta...
et al. (2010) reported that the number of protozoa increased by tannin supplementation in sheep that consumed a concentrated diet. It might be related to adaptation factors and nutrient composition in the diet.

Cieslak et al. (2013) reported that tannins may inhibit growth, and affect development and activity of the population of methanogens indirectly (by reducing the number of protozoa associated with methanogens) and directly (by affecting methanogens). Tannin might also increase propionate production by affecting methanogens through reduced competition for hydrogen utilization. Moreover, the insignificant protozoa population or methane production on addition of natural crude tannin from neem leaves might be due to low dosage of the tannin. Bhatta et al. (2014) revealed that A. indica leaves decreased methane or protozoa number with the minimum dosage of 25% in dry matter basis.

Based on Figure 2, the effectiveness of supplementing IM (T1), IM + tannin leaves (T4), and OM (T2) with higher gas production than monensin and control indicated that either tannin with organic or IM potential in replacing monensin. However, in this result monensin treatment was similar to control as well as the previous study reported by Smith et al. (2010) that VFA and methane in vitro production could not be influenced by addition of monensin up to 0.6 mg/L. Aderinboye et al. (2012), monensin had also potential to increase feed digestibility by increasing propionate proportion that could be associated with reduction of methanogenesis activity. Feed digestion improvement was related to the reduction in methane followed by increase in NGR value (Figure 1). Although the treatment was not affected by in vitro methane production, supplementation of A. indica leaves should be evaluated by an in vivo experiment which is related to the complexity processes in ruminal digestion.

| Variables | T0 | T1 | T2 | T3 | T4 | T5 | T6 | SEM | P-value |
|-----------|----|----|----|----|----|----|----|-----|---------|
| IVOMD (%) | 63.26 | 63.67 | 62.67 | 60.44 | 63.64 | 61.27 | 60.55 | 0.733 | 0.831 |
| Gp (ml)  | 69.58<sup>a</sup> | 74.08<sup>b</sup> | 76.50<sup>b</sup> | 71.58<sup>a</sup> | 78.25<sup>b</sup> | 66.00<sup>a</sup> | 70.58<sup>b</sup> | 1.000 | 0.003 |
| B (ml)   | 79.76<sup>a</sup> | 83.44<sup>b</sup> | 85.16<sup>b</sup> | 80.99<sup>a</sup> | 87.56<sup>b</sup> | 76.69<sup>b</sup> | 79.38<sup>a</sup> | 0.883 | 0.001 |
| k (ml/h) | 0.0460<sup>a</sup> | 0.0480<sup>b</sup> | 0.0510<sup>b</sup> | 0.0476<sup>a</sup> | 0.0500<sup>b</sup> | 0.0430<sup>a</sup> | 0.0487<sup>b</sup> | 0.001 | 0.038 |
| L (h)    | 0.557 | 0.472 | 0.469 | 0.582 | 0.506 | 0.317 | 0.570 | 0.037 | 0.640 |
| VFA (mM) | 202.44 | 221.78 | 268.78 | 214.87 | 249.48 | 200.74 | 226.43 | 7.643 | 0.109 |
| C<sub>2</sub> (mM) | 146.46 | 160.68 | 192.63 | 157.31 | 178.29 | 145.87 | 164.61 | 5.338 | 0.152 |
| C<sub>3</sub> (mM) | 38.05 | 39.90 | 48.06 | 38.55 | 45.30 | 36.10 | 41.47 | 1.331 | 0.139 |
| C<sub>4</sub> (mM) | 17.93<sup>a</sup> | 21.19<sup>a</sup> | 28.09<sup>b</sup> | 19.01<sup>a</sup> | 25.89<sup>b</sup> | 18.77<sup>a</sup> | 20.36<sup>a</sup> | 1.067 | 0.007 |
| C<sub>2</sub>/C<sub>3</sub> | 3.86 | 4.03 | 4.01 | 4.08 | 3.94 | 4.03 | 3.97 | 0.998 | 0.337 |
| NH<sub>3</sub>(mg/100 ml) | 4.84 | 4.85 | 6.01 | 6.12 | 6.23 | 5.04 | 5.92 | 0.344 | 0.283 |
| Protozoa (×10<sup>5</sup> cells/ml) | 2.68 | 2.98 | 4.18 | 3.10 | 3.15 | 3.65 | 3.15 | 0.194 | 0.535 |

T0 (<i>P. hybrid</i> as a control), T1 (T0 + 3% inorganic mineral/IM), T2 (T0 + 3% organic mineral/OM), T3 (T0 + 2% A. indica/AI), T4 (T0 + 3% IM + 2% AI), T5 (T0 + 3% OM + 2% AI), and T6 (T0 + 40 ppm monensin). B: maximum gas production, k: rate of gas production, L: lag time, Gp: cumulative gas production at 48 h. IVOMD: in vitro organic matter digestibility. If the superscript in same row differs it means significant differences (<i>P</i> < .05). SEM: standard error mean.

Figure 1. Relationship of methane production and non-glucogenic ratio affected by treatments.
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Aderinboye RY, Onwuka CFI, Arigbede OM, Oduguwa OO, Aina ABJ. 2012. Effect
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4. Conclusion
Supplementation of organic or IM improved in vitro fermentability of forage (based on total gas production, total VFA, and butyrate concentration) while their combination with tannin-containing leaves reduced the fermentability without affecting methane production. OM could be considered for use as an additive to support rumen fermentation.

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Figure 2. Kinetics curve of gas production from king grass treated by an/oranic mineral, tannin, and monensin. T0 (P. hybrid as a control), T1 (T0 + 3% inorganic mineral/IM), T2 (T0 + 3% organic mineral/OM), T3 (T0 + 2% Azadirachta indica/ All), T4 (T0 + 3% IM + 2% AI), T5 (T0 + 3% OM + 2% AI), and T6 (T0 + 40 ppm Monensin). Different letters, a, b, c, mean significant differences (P < 0.05).