

Effect of napier grass supplemented with *Gliricidia sepium*, *Sapindus rarak* or *Hibiscus rosa-sinensis* on *in vitro* rumen fermentation profiles and methanogenesis

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ABSTRAK

Penelitian ini menguji efek suplementasi daun gamal (DG, *Gliricidia sepium*), buah lerak (BL, *Sapindus rarak*), atau daun kembang sepatu (DKS, *Hibiscus rosa-sinensis*) pada rumput gajah (RG, *Pennisetum purpureum*) terhadap fermentasi rumen dan metanogenesis secara *in vitro*. Rancangan percobaan yang dilakukan adalah rancangan acak kelompok dengan tujuh perlakuan dan empat ulangan. Perlakuan yang diujikan meliputi: RG, DG, BL, DKS, RG 70% + DG 30%, RG 70% + BL 30%, dan RG 70% + DKS 30%. Peubah yang diamati adalah kandungan nutrisi, produksi gas, pencernaan, profil *volatile fatty acid* (VFA), amonia dan populasi mikroba rumen. Data kemudian dianalisis menggunakan analisis ragam (ANOVA) dan dilanjutkan dengan uji Duncan untuk membandingkan antar perlakuan. Hasil menunjukkan bahwa suplementasi pakan mengandung saponin seperti LF atau HL pada NG tidak mengubah produksi gas pada waktu inkubasi 24 dan 48 jam. Pakan HL baik tunggal maupun kombinasi dengan NG menghasilkan pencernaan bahan organik tertinggi dibandingkan dengan perlakuan lainnya ($P < 0.05$). Suplementasi LF menurunkan emisi gas metana dibandingkan dengan NG tunggal ($P < 0.05$), serta mengubah profil VFA menjadi lebih tinggi propionat dan lebih rendah asetat ($P < 0.05$). Suplementasi LF cenderung menurunkan populasi protozoa rumen namun tidak mengubah populasi metanogen. Dapat disimpulkan bahwa penggunaan pakan tinggi saponin bermanfaat dalam menurunkan emisi gas metana di dalam rumen.

Kata kunci: *In vitro*, fermentasi rumen, produksi gas, saponin, pencernaan

ABSTRACT

This study examined the supplementation effects of gliricidia leaves (GL, *Gliricidia sepium*), lerak fruit (LF, *Sapindus rarak*), or hibiscus leaves (HL, *Hibiscus rosa-sinensis*) on *in vitro* rumen fermentation and methanogenesis and made a comparison with the Napier grass (NG, *Pennisetum purpureum*) grass. *In vitro* rumen fermentation was designed according to a randomized complete block design with four replications and seven treatments: NG, GL, LF, HL, NG 70% + GL 30%, NG 70% +

LF 30% and NG 70% + HL 30%. The generated data were subjected to analysis of variance (ANOVA) with Duncan's multiple range test and compared among treatment means. Addition of a plant containing saponin such as LF or HL to NG did not alter gas production after 24 and 48 h of incubation period in comparison to NG alone. The HL alone or blended with NG produced the highest IVOMD during the fermentation process as compared to other treatments ($P < 0.05$). Adding LF supplement either singly or in combination significantly ($P < 0.05$) reduced methane production in terms of %TVFA as compared to NG. Supplementation of LF plants has shifted VFA proportion towards more propionate and less acetate. The microbial population of LF, whether single or in combination had a tendency to reduce the rumen protozoa population but had no effect on methanogen population. It can be concluded that utilization of saponin-rich materials is particularly beneficial for reducing ruminal methane emission.

Keywords: *In vitro*, rumen fermentation, gas production, saponins, digestibility.

INTRODUCTION

Napier grass (*Pennisetum purpureum*) is a widely used forage species for ruminant feeding in the tropics. It is known to have high nutritive value among grass species and therefore is viewed as a suitable perennial fodder ideal for intensively managed livestock production systems. The commonly practiced feeding management is cut-and-carry. However, a diet of napier grass alone is nutritionally insufficient, particularly protein requirements of ruminants. As such, supplementation of napier grass with other high protein feedstuffs such as gliricidia leaves (*Gliricidia sepium*) is necessary to close the gap between nutrients available in feeds and nutrient requirements of the animals. Gliricidia is a leguminous tree widely used in the semi-arid and sub-humid tropics containing high protein content, low fiber, and highly digestible. This medium-sized plant grows abundantly and is able to preserve its high quality even at the height of the dry season. It is extensively utilized in the cattle-rearing industry as a natural defence and source of nutrition during dry seasons (Wood *et al.*, 1998). The plant has been known to be potentially good for sustaining increased livestock production (National Academy of Sciences, 1977) and potential to enhance the productivity and sustainability of agricultural systems (Dawson *et al.*, 1996).

On the other hand, feeding of high-fiber feedstuffs such as grasses and agricultural residues generally results in a high enteric methane emission. Methane is among the main sources of greenhouse gases in the atmosphere (Tian *et al.*, 2016) and livestock is a key source of greenhouse gas emissions, particularly methane. Enteric fermentation of feed by ruminants is one of the most substantial sources of anthropogenic

methane emissions, i.e. as much as 25% of the total methane emissions (Hristov *et al.*, 2017). Besides its significant contribution to global warming, methane emission from ruminants also results in the loss of approximately 5-9% of dietary gross energy (Jeyanathan *et al.*, 2014) that would otherwise be used to support productivity.

An approach to mitigate enteric methane emission from ruminants is by using natural compounds such as tannin, saponin, and essential oil. Some efforts have been made and been proved to be effective in lowering the methane emission (Cottle *et al.*, 2011). Methane from ruminants may be notified by type of diet as a result of the disparity in their chemical make up or the existence of some plant secondary metabolites (saponins, tannins, etc.) or other unknown constituents in feeds reported earlier by Prusty *et al.* (2014). Outcomes of plant secondary metabolites or extracts on methane emissions have been earlier documented by Kumar *et al.* (2014). Natural compounds are favoured (Kondo *et al.*, 2014), particularly after the use of antibiotics was banned as feed additives in several countries. Plants originating from the tropics are, in general, high in these natural compounds including saponin. Lerak fruits (*Sapindus rarak*) and hibiscus or rose mallow leaves (*Hibiscus rosa-sinensis*) have been reported to contain considerable amounts of saponin (Wina *et al.*, 2006), which comprise aglycon (steroid or triterpenoid) and glycon structures (Cheok *et al.*, 2014). This structure provides saponin with a membranolytic activity (Wojciechowski *et al.*, 2016) and clarifies their role in reducing methane emission *in vitro* (Rira *et al.*, 2015), therefore possessing the potential to be used as methane mitigating agents.

The aim of this study was to evaluate the partial replacement of gliricidia leaves, lerak

fruits or hibiscus leaves in napier grass, especially with regard to their influences on methanogenesis and rumen fermentation *in vitro*.

MATERIALS AND METHODS

Determination of Chemical Composition

Feed samples were subjected to analysis for contents of dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), ash in line with AOAC procedure (AOAC, 1990). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were evaluated by following Van Soest *et al.* (1991). Analysis of total saponin was done in accordance with the method of Hiai and Nakajima (1976) and calibrated against diosgenin standard (Sigma-Aldrich D1634, Sigma Aldrich Chemie GmbH, Steinheim, Germany).

In vitro Rumen Fermentation Procedure

Samples of napier grass (NG), gliricidia leaves (GL), lerak fruits (LF) and hibiscus leaves (HL) were subjected to *in vitro* incubation by using buffered rumen fluid (Menke and Steingass, 1988). The experimental treatments were: NG, GL, LF, HL, NG 70% + GL 30%, NG 70% + LF 30%, and NG 70% + HL 30%. Rumen fluid was sourced from two fistulated Brahman-cross cattle prior to the morning feed, filtered and mixed with buffer solution in ratio (rumen fluid: buffer) 1:4 v/v. The incubation was performed in four replicates by employing a randomized complete block design. Total gas generated was noted at 0, 3, 6, 9, 12, 24, 30, 36, and 48 h following incubation employing a gas syringe. The kinetic parameters of *in vitro* cumulative gas produced were estimated as per Ørskov and McDonald (1979).

The pH of rumen fluid samples was also noted with a pH meter while ammonia (NH₃) concentration was determined according to Parsons *et al.* (1984). Volatile fatty acids (VFA) were determined by employing a gas chromatograph (Hewlett Packard 6890 GC system) according to the procedure of Cottyn and Boucque (1968). Amount of methane produced was computed by employing the equation of Moss *et al.* (2000). The isolation of DNA from the rumen fluid sample was done with the use of CTAB DNA extraction (Neumann *et al.*, 1992). The rumen microbial populations analyzed were total bacteria, methanogens, total protozoa, *Fibrobacter succinogenes*, *Ruminococcus albus*

and *Ruminococcus flavefaciens* using BioRad CFX96 real-time PCR system. The real-time cycling condition was prepared as described by Muideen *et al.* (2017).

Statistical Analysis

The experiment on *in vitro* rumen fermentation used a randomized complete block design with four replicates, and with two incubation syringes representing each replicate. Different lots of rumen fluid (runs) made up the block. The obtained data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Crude protein contents of experimental diets varied from 3.55% in LF to 22.2% DM in GL (Table 1). Such high CP observed in GL confirms its use as a protein supplement. Crude fiber contents differed from 1.15% to 35.3% DM, and similar with those of NDF, ADF and ADL contents. Both LF and HL contained a considerable amount of saponin.

Total gas production of GL, LF or HL, either individually or combined with NG is presented in Table 2. Addition of a plant containing saponin such as LF or HL to NG did not alter gas production after 24 and 48 h of incubation period in comparison to NG alone. During the *in vitro* fermentation process, the gas is produced as a result of substrates metabolism by the rumen microbes. Total gas production *in vitro* increased dramatically within the first 10 h of incubation and started to slow down because of the reduction of fermentable substrates. The decreasing quantity of fermentable substrates has been reported previously (Jayanegara *et al.*, 2006). Different with the current finding, Herdian *et al.* (2011) reported that the gas production decreased significantly by supplementing the NG with *Morinda citrifolia*, a saponin-containing plant. By doing so, the saponin level increases from 5% to 10% of DM feed and consequently reduces the protozoa population and lowers the gas production (Teferedegne *et al.*, 1999).

Gas production is essentially caused by microbial fermentation of carbohydrates to various volatile fatty acids in the rumen such as acetate, propionate and butyrate (Steingass and Menke, 1986), and considerable changes in carbohydrate fractions were evidenced by the total gas generated. *In vitro* OMD also varied among

Table 1. Chemical Composition of Experimental Diets (% Dry Matter)

Diet	DM	OM	CP	EE	CF	NDF	ADF	ADL	Ash	Saponin
NG	95.2	84.5	9.06	1.41	35.3	78.1	36.4	21.9	4.80	nd
GL	88.3	91.3	22.2	4.17	12.4	55.8	27.2	18.8	8.69	nd
LF	95.5	89.7	3.55	0.57	1.15	17.5	16.7	5.33	3.25	36.4
HL	97.6	88.1	18.5	4.24	1.55	31.9	31.0	23.5	11.9	16.6
NG 70% + GL 30%	94.0	86.5	13.0	2.23	28.4	71.4	33.6	21.0	5.97	nd
NG 70% + LF 30%	95.3	86.1	7.41	1.16	25.0	59.9	30.7	16.9	4.34	10.9
NG 70% + HL 30%	95.9	85.6	11.9	2.26	25.2	64.3	34.8	22.4	6.93	4.98

NG: napier grass, GL: gliricidia leaves, LF: lerak fruits, HL: hibiscus leaves, DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, CF: crude fiber, NDF: neutral detergent fiber, ADF: acid detergent fiber, ADL: acid detergent lignin, nd: not detected.

Table 2. Effect of Experimental Diets on *In vitro* Gas Production

Diet	Gas 24 h (mL)	Gas 48 h (mL)	<i>b</i> (mL)	<i>c</i> (1/h)	IVOMD (%)
NG	22.4±8.27 ^{abc}	31.5±6.54 ^b	36.1±8.25 ^b	0.01±0.01	42.0±7.35 ^{bc}
GL	16.5±6.67 ^a	20.9±8.07 ^a	24.7±9.91 ^a	0.05±0.01	45.2±5.92 ^{cd}
LF	17.6±11.28 ^{ab}	22.7±13.4 ^a	37.6±15.0 ^b	0.04±0.02	34.2±10.0 ^a
HL	30.7±8.32 ^d	36.9±7.60 ^b	38.5±4.71 ^b	0.07±0.03	58.3±7.40 ^e
NG 70% + GL 30%	23.9±6.25 ^{bc}	31.8±8.92 ^b	38.8±8.30 ^b	0.04±0.03	45.8±5.59 ^{cd}
NG 70% + LF 30%	19.4±9.73 ^{ab}	30.0±5.35 ^b	36.3±3.77 ^b	0.04±0.01	38.2±8.66 ^{ab}
NG 70% + HL 30%	28.6±7.22 ^{cd}	33.3±4.73 ^b	36.5±5.35 ^b	0.06±0.02	50.8±6.42 ^d
SEM	0.877	1.251	1.25	0.07	8.166
P-value	<0.001	0.002	0.003	0.237	<0.001

Different superscripts within the same column are statistically different at P<0.05.

NG: napier grass, GL: gliricidia leaves, LF: lerak fruits, HL: hibiscus leaves, *b*: potential gas production, *c*: gas production rate constant, IVOMD: *in vitro* organic matter digestibility, SEM: standard error of mean

the experimental diets in this study (P<0.05). The HL alone or blended with NG produced the highest IVOMD during the fermentation process as compared to other treatments (P<0.05). It is generally accepted that any feedstuffs that would give higher gas production and IVOMD have a tendency to produce higher CH₄ per gram DM

incubated (Jayanegara *et al.*, 2011). In general, the digestibility of Napier grass is affected by several factors, for instance, the cultivar selection and the management practices (Zailan *et al.*, 2015). The asymptotic gas production (*b*) ranged from 24.7 to 38.8 mL, and the rate of production (*c*) from 0.01 to 0.07 h⁻¹. The highest value of asymptotic gas

production was observed in HL (38.5 mL) among the single incubated feedstuffs. The cell wall contents (NDF and ADF) were inversely related with gas production at all incubation times and b parameter except the rate of gas production (c). The negative correlation between gas production and the cell wall contents may be caused by the decrease of microbial activity. This result is in line with findings of Abdulrazak *et al.* (2000). In this study, we agree with the report by Khazaal *et al.* (1993), Blummel and Orskov (1993), who noted a connection between DM disappearance and gas production, but they failed to identify a noteworthy association between the gas production rates (c).

The rumen pH values indicated a normal rumen condition, ranging from 6.8 to 7.1. Supplementation of GL, LF or HL had no noteworthy impact on rumen pH (Table 3). These results are in agreement with those reported by Hess *et al.* (2003) who also failed to find any noteworthy alterations in the pH of the ruminal liquor by the effect of the pericarp and the entire fruit of *Sapindus saponaria*. Increased rumen pH favours the development and activity of cellulolytic bacteria, thus improving fiber digestion, DM intake and development performance (Zinn *et al.*, 1999). Supplementation of saponin-rich plants to NG did not alter the NH₃ concentration in comparison with the control treatment. The range of NH₃ in the current study

ranged from 19.1 to 22.2 mM, which was a little higher than the optimum concentration to support microbial protein synthesis based on McDonald *et al.* (2002) i.e. 6-21 mM. A tendency of lower ammonia concentration in LF and NG + LF combination could be because of the anti-protozoal activity of LF. The reduced number of protozoa leads to lower bacterial lysis and thus reduced release of protein breakdown products. The well-documented effects of saponins on protozoal action and the following reduction in bacterial protein turnover (Wallace, 2004) could help in explaining the impact of saponins on rumen ammonia levels, but it is still unclear to what extent saponins disrupt the dietary protein breakdown. However, Muetzel *et al.* (2003) stated that saponins from sesbania did not prevent feed protein from being degraded *in vitro*. Apart from saponin, different protein fractions present in the feed materials also determine ammonia concentration in the rumen (Jayanegara *et al.*, 2016).

Adding saponin supplement either singly or in combination significantly (P<0.05) reduced methane production in terms of %TVFA as compared to NG. Saponins may lower methane emission by eliminating the rumen protozoa population because of the capacity of saponins to bind the sterol in the protozoa cell membranes, resulting in cell lysis (Hristov *et al.*, 1999). Methane emissions have a close relationship with

Table 3. Effect of Experimental Diets on Ruminal pH, Ammonia (NH₃) and Methane (CH₄) Formation

Diet	pH	NH ₃ (mM)	CH ₄ (% TVFA)
NG	7.05	20.7	36.9 ^c
GL	7.08	22.2	36.3 ^{bc}
LF	7.07	19.1	35.1 ^a
HL	6.87	21.3	36.8 ^c
NG 70% + GL 30%	6.94	20.0	36.7 ^c
NG 70% + LF 30%	6.98	19.2	36.1 ^{ab}
NG 70% + HL 30%	6.81	21.3	36.9 ^c
SEM	0.059	0.442	0.150
P-value	0.254	0.374	<0.001

Different superscripts within the same column are significantly different at P<0.05.

NG: napier grass, GL: gliricidia leaves, LF: lerak fruits, HL: hibiscus leaves, TVFA: total volatile fatty acids, SEM: standard error of mean.

the quantum of rumen fermented OM or the quantum of digestible OM as more than half of digestion takes place in the rumen. Since the proportion of the individual VFAs is affected by the OM composition of the diet, mostly by the nature and rate of fermentation of carbohydrates, these dietary features will have substantial effect on methane production. Starch rich diets encourage propionate production, lowers the methane production/fermented OM ratio in the rumen. In contrast, a roughage-based diet will enhance the ratio (Moss *et al.*, 2000).

The effects of experimental diets on ruminal VFA profiles are shown in Table 4. There were no significant differences in total VFA among the diets. Supplementation of saponin-rich plants has shifted VFA proportion towards more propionate and less acetate. This kind of shift in VFA proportion due to saponin supplementation of ruminant animal has been reported earlier (Makkar *et al.*, 1995). A number of studies have noted higher propionate production at the expense of acetate and butyrate production, whereas others have observed no effect. Part of this change is definitely due to differences in saponin type and intensity, but it is interesting to note that the effect

appears to be more marked (increase in propionate) in the case of a diet rich in grain or starch is used (Suharti *et al.*, 2011) contrary to fibre-rich diets or mixed forages (Hess *et al.*, 2003). This indicates that the diet may impact the response to saponin supplementation. Propionate proportion with the addition of LF supplementation neither single nor in combination with NG shows enhanced fermentation efficiency by rumen microbes. These results are supported by Suharti *et al.* (2011) who found a significant increase of propionate proportion following saponin extract addition. The higher propionate production lower the supply of H₂ as the production of propionate in the metabolic pathway in the rumen utilises H₂, which is in competition with methanogenic bacteria in the formation of methane. As such, the use of LF as a supplement holds great promise for the purpose of reducing methane production in the rumen. Earlier research has reported that tea saponin at a level of 0.5% DM considerably raised propionate, lowered acetate proportion and reduced methane emissions (Beauchemin *et al.*, 2008).

The microbial population of LF, whether single or in combination had a tendency to reduce

Table 4. Effect of Experimental Diets on Ruminal Volatile Fatty Acid Profiles

Diet	TVFA (mM)	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
.....%							
NG	53.6	84.5 ^d	9.18 ^a	0.76	3.91	1.08 ^{ab}	0.57
GL	50.6	82.9 ^{bc}	10.27 ^a	1.17	3.31	0.41 ^{ab}	0.48
LF	55.7	81.7 ^a	11.54 ^b	1.51	3.75	0.51 ^a	0.56
HL	51.8	84.4 ^d	9.32 ^a	0.68	3.61	0.56 ^b	0.65
NG 70% + GL 30%	53.4	84.6 ^d	9.96 ^a	0.65	3.41	0.94 ^a	0.48
NG 70% + LF 30%	51.1	82.9 ^b	11.50 ^b	0.76	3.59	0.74 ^a	0.48
NG 70% + HL 30%	50.6	84.2 ^d	9.28 ^a	0.77	3.97	1.11 ^{ab}	0.64
SEM	2.59	0.200	0.222	0.097	0.113	0.053	0.024
P-value	0.991	<0.001	<0.001	0.101	0.060	0.010	0.133

Different superscripts within the same column are significantly different at P<0.05.

NG: napier grass, GL: gliricidia leaves, LF: lerak fruits, HL: hibiscus leaves, TVFA: total volatile fatty acids, SEM: standard error of mean

Table 5. Effect of Experimental Diets on Ruminal Microbial Population (log cell/mL)

Feed	Total bacteria	<i>Ruminococcus flavefaciens</i>	<i>Ruminococcus albus</i>	<i>Fibrobacter succinogenes</i>	Total protozoa	Methanogen
NG	10.5	5.42 ^{bc}	7.23	6.56	5.73	7.06
GL	10.7	4.46 ^{ab}	7.20	6.27	5.76	6.90
LF	10.6	5.63 ^{bc}	7.11	6.46	4.59	7.06
HL	10.7	5.52 ^{bc}	6.93	6.75	6.37	6.97
NG 70% + GL 30%	10.7	5.77 ^{bc}	7.47	6.55	4.23	7.11
NG 70% + LF 30%	10.6	4.08 ^a	7.14	6.19	5.25	6.99
NG 70% + HL 30%	10.8	5.97 ^c	7.61	6.72	5.62	7.28
SEM	0.063	0.186	0.089	0.248	0.154	0.052
P-value	0.754	0.033	0.413	0.892	0.318	0.619

Different superscripts within the same column are significantly different at $P < 0.05$.

NG: napier grass, GL: gliricidia leaves, LF: lerak fruits, HL: hibiscus leaves, SEM: standard error of mean

the rumen protozoa population (Table 5). Reducing protozoal counts by supplementing with saponins-rich extract (Patra *et al.*, 2006) or pod and seed (Wanapat *et al.*, 2013) or fruits (Hess *et al.*, 2003) has been reported previously. The effect of supplemented and non-supplemented saponins, either single or in combination with NG did not significantly reduce the rumen total bacteria population in the present study. Our data are in congruence with Wanapat *et al.* (2013) who reported the composition of total bacteria and genera ciliate protozoa populations had no effect on the herbs supplemented group. Methanogen population, in contrast to our expectation, was not altered by supplementation of saponin-rich materials.

The number of *R. flavefaciens* tended to increase in the blend with HL in the present study. Muetzel *et al.* (2003) also noted an increase in *R. flavefaciens* which is one of the predominant fiber-degrading bacteria (Wang *et al.*, 2000) when tested with saponin-containing *S. pachycarpa* leaves *in vitro*. The population of the predominant fiber-degrading bacteria resulted from LF diet is in agreement with other findings using yucca saponin (Ningrat *et al.*, 2002). *Fibrobacter succinogenes* was not affected, but *Ruminococcus albus* and *R. flavefaciens* were

practically unable to degrade cell wall components in the presence of yucca saponins. The authors arrived at the conclusion that yucca saponin had more adverse effect on the Gram-positive bacteria as compared to those of the Gram-negative bacteria. Muetzel *et al.* (2003) also reported that *S. pachycarpa* did not affect *F. succinogenes* (Gram-negative). Another study in pure culture demonstrated that the development of cellulolytic bacteria was reduced to a small extent when lerak extract was added in the culture (Ningrat *et al.*, 2002).

CONCLUSION

In general, using saponin-containing plant (*Hibiscus rosa-sinensis*) alone or blended with Napier grass produces the highest IVOMD during the fermentation process. Incorporation of 30% lerak fruit decreases methane emission, and alters VFA profiles toward more propionate at the expense of acetate.

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