SCREENING APPROACHES FOR METHANE MITIGATING POTENTIAL OF TANNIN-CONTAINING PLANTS UNDER *IN VITRO* RUMEN ENVIRONMENT

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ABSTRAK

Tujuan penelitian ini adalah untuk melakukan pemilahan terhadap hijauan mengandung tanin yang memiliki sifat menurunkan emisi gas metana dengan menggunakan pendekatan univariabel, bivariabel dan multivariabel (*principal component analysis*, PCA). Sampel hijauan berasal dari beberapa negara, yakni Indonesia (n = 27 spesies), Mongolia (n = 14), Swiss (n = 16) dan Jerman (n = 3). Hijauan diinkubasi secara *in vitro* dengan cairan rumen-buffer pada suhu 39^{o}C selama 24 jam. Total produksi gas diamati sebagai indikator kualitas hijauan dan emisi gas metana diukur. Hasil menunjukkan bahwa, berdasarkan pendekatan bivariabel, hijauan yang menghasilkan emisi metana rendah umumnya juga memiliki kualitas yang kurang baik atau rendah total produksi gasnya, kecuali *Rhus typhina* yakni sebesar 43 ml metana/200 mg bahan kering. *Loading plot* dari PCA menunjukkan bahwa semua fraksi fenolik hijauan berlawanan arah dengan gas metana dan total produksi gas. Hijauan yang berlawanan arah dengan produksi metana adalah *Bergenia crassifolia*, *Swietenia mahagoni*, *Clidemia hirta*, *Peltiphyllum peltatum*, *Acacia villosa* dan *R. typhina*. Dapat disimpulkan bahwa, untuk hijauan mengandung tanin, pemilahan berdasarkan pendekatan univariabel, bivariabel dan multivariabel terkait emisi gas metana menunjukkan hasil yang serupa.

Kata kunci : Metana, kualitas hijauan, pemilahan, korelasi, tanin, fenolik

ABSTRACT

The aim of the present study was to conduct univariate, bivariate and multivariate (principal component analysis, PCA) approaches in the screening of tannin-containing plants from various collection sites for their CH_4 mitigating properties. Plant samples were obtained from various collection sites in different countries, i.e. Indonesia (n = 27 species), Mongolia (n = 14), Switzerland (n = 16) and Germany (n = 3). The plants were incubated in vitro with buffered-rumen fluid at 39^{o}C for 24 h. Total gas production was recorded as an indicator of feed quality and emission of CH_4 was measured. Results showed that, based on bivariate screening, generally, plants possessed low CH_4 production had low quality or low total gas production except Rhus typhina, i.e. 43 ml/200 mg DM. The loading plot of PCA showed that all phenolic fractions were in the opposite direction with CH_4 and total gas production. Plants clustered together in reverse direction to that of CH_4 were Bergenia crassifolia root and leaf, Swietenia mahagoni, Clidemia hirta, Peltiphyllum peltatum, Acacia villosa and R. typhina. It was conluded that, for tannin-containing plants, screenings based on univariate, bivariate and multivariate approaches in relation to ruminal CH_4 emission led to similar results

Keywords : Methane, forage quality, screening, correlation, tannin, phenolic

INTRODUCTION

Forage quality is a determining factor for the productivity of ruminants. Several important properties related to forage quality are energy content, fiber, protein, anti-nutritional compounds and the digestibility of forage in the digestive tract. On the other hand, the concern on global warming due to accumulation of green-house gases has increased in the last decades, including methane (CH_4). Ruminants are among major contributors of atmospheric CH_4 which is generated from microbial fermentation in the rumen (Moss *et al.*, 2000), and may be more prevalent in the tropical area. It has been estimated that ruminants produce 80 million tons of CH_4 annually which accounts for 28% of total anthropogenic CH_4 emissions (Beauchemin *et al.*, 2008). Such emission is not only associated with environmental problem but also reflects the loss of energy from animal and, hence, may reduce the amount of energy for production purposes. Up to 12% of the gross energy from feed consumed by ruminants is lost as CH_4 (McCrabb and Hunter, 1999).

The above-mentioned factors have led to the exploration of forages that are not only good in quality but also are able to reduce CH_4 formation in the rumen. Tannin-containing forages or extracts have been reported to be able to reduce CH_4 emission from ruminants, both in *in vitro* and *in vivo* experiments (e.g., Tavendale *et al*., 2005; Tiemann *et al*., 2008; Bhatta *et al*., 2009). However, further research still needs to be conducted to find most promising tannin-containing plants, i.e. effective in mitigating CH_4 emission at simultaneously of high quality. Screenings of plants that possess CH_4 mitigating properties have been done using different approaches, such as using univariate approach (Soliva *et al*., 2008), bivariate (Bodas *et al*., 2008; Garcia-Gonzalez *et al*., 2008) and multivariate (Jayanegara *et al*., 2011a). However, no studies so far have attempted to address these different approaches simultaneously when screening the plants and to compare the results obtained.

Based on this background, therefore, the aim of the present study is to conduct univariate, bivariate and multivariate approaches in the screening of tannin-containing plants from various collection sites for their CH_4 mitigating properties. A main point of interest is to observe whether these different approaches will lead to a different conclusion when screening the plants. Additionally, as a second objective, this study is aimed at observing the relationships between plant phenolic fractions and CH_4 emission using an extended database of plants collected from various sites, i.e. Indonesia, Mongolia, Switzerland and Germany.

MATERIALS AND METHODS

Plant Samples

Plant samples were obtained from various collection sites in different countries, i.e. Indonesia (n = 27 species), Mongolia (n = 14), Switzerland (n = 16) and Germany (n = 3). The data were based on some previous studies, i.e. Jayanegara *et al*. (2009a) for the plants from Mongolia and Germany, Jayanegara *et al*. (2011a) for the plants from Indonesia, and Jayanegara *et al*. (2011b) for the plants from Switzerland. Plant samples from Indonesia consisted of one grass, four herbs, nine shrubs and 13 tree species; all of them are either commonly used as ruminant feeds in rural areas or as traditional veterinary medicinal plants. The plants collected from Mongolia are used locally in the region of production as medicinal plants. Plants from Switzerland were obtained from the region of the Alps at altitudes of 800-2300 m above sea level, consisted of two grasses, eight non-leguminous herbs, three herbaceous legumes and three tree species. The majority of these plants is used for grazing of ruminants during European summer period, particularly for dairy production. The plants from Germany were collected from the Botanical Garden of the University of Hohenheim in Stuttgart. All plant samples were oven dried at 50-60^oC and ground to pass 1 mm sieve. Each plant was encoded by its country of origin, i.e. I, M, S and G for plants obtained from Indonesia, Mongolia, Switzerland and Germany, respectively, and numbered according to alphabetical order within country.

Chemical Composition and *In Vitro* Procedures

The plant samples were analyzed for their chemical composition. These included crude protein (CP), ether extract (EE; AOAC, 1997), neutral detergent fiber (NDF), acid detergent fiber (ADF; Van Soest *et al*., 1991), total phenols (TP), non-tannin phenols (NTP), total tannins (TT), condensed tannins (CT; Makkar, 2003a) and hydrolysable tannins (HT; Singh *et al*., 2005). The plants were incubated *in vitro* with buffered-rumen fluid by using the Hohenheim gas test method (Menke and Steingass, 1988), incubated at 39^oC for 24 h. In addition, standard hay and concentrate (obtained from the Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany) were incubated, serving as control for the successful incubation. After the incubation, total gas production was recorded as an indicator of feed quality. Emission of CH_4 was measured by using an infrared methane analyzer (Goel *et al*., 2008) or a gas chromatograph (Soliva and Hess, 2007). These two different methods result in a similar CH_4 value of hay standard, i.e. between 160-170 ml/l gas.

Statistical Analysis

Data of all plants from various collection sites were tabulated into a database. Minimum and maximum values for each variable were recorded, and mean and standard deviation were calculated. Pearson's correlation analysis was performed to the data, i.e. the chemical composition and fermentation variables (total gas production and CH_4 emission). Screening approaches were based on univariate (CH_4 only), bivariate (total gas and CH_4) and multivariate (all variables investigated) using the principal component analysis (PCA). For the univariate approach, the plants considered were those which produced $CH_4 \leq$ average CH_4 across all plants minus 1 × standard deviation. For the bivariate approach, the screening was based on a two-dimensional plot between total gas and CH_4. For the multivariate approach, a PCA was applied to the data with Kaiser's criterion, i.e. Eigenvalue ≥ 1.0 to extract the principal components (PC) without rotation method. This procedure generated factor loading and factor score. The first two PC were plotted both for the loading and the score. The loading plot is used for describing the relationship among variables and the score plot is used for classifying the plants according to the loading. All statistical analyses were conducted using SPSS version 17.0 (2008) and the figures were generated using SigmaPlot version 11.0 (2008).

RESULTS

Chemical Composition

Summary of chemical composition of the plants investigated is presented in Table 1. Plant samples collected from Indonesia contained the highest CP contents (on average basis) compared to from other collection sites. Very high CP contents were found in the leaves of *Carica papaya* (386 g/kg DM; code I7) and *Manihot esculenta* (377 g/kg DM; I17) across all plants. All plants contained low EE with the average less than 35 g/kg DM from the four collection sites. Plants from Mongolia contained higher ADF than the others. Quite a diverse TP contents were found across all collection sites, except those from Switzerland. The average of TP in these plants was 31 g/kg DM with the highest was found in *Hedysarum hedysaroides*, i.e. 69 g/kg DM (S8). This pattern was similarly observed for TT, CT and HT. Several plants, i.e. *Rhus typhina* (G2), *Acacia villosa* (I2) and *Clidemia hirta* (I8) contained TT more than 200 g/kg DM. Very high CT contents were found in *Vaccinium vitis idea* (M14) and *Swietenia mahagoni* (I27) with the values of 175 and 86 g/kg DM, respectively.

Relationships between Variables

Variables CP and EE were unrelated to total gas production and CH_4 (Table 2). Both NDF and ADF were positively correlated (both at $P<0.001$) with CH_4 emission, however, only ADF was negatively correlated with total gas ($P<0.05$). All phenolic fractions were negatively correlated with CH_4 by following the order of magnitude: TP > TT > HT > NTP > CT. The phenolics were also negatively correlated with total gas production.

Screening of Plants

Across all plants, the average CH_4 emission after 24 h *in vitro* incubation was 137 (±32) ml/l gas (Table 1). Therefore, for the screening based on univariate approach, the promising plants were those which produced $CH_4 \leq 105$ ml/l gas. These plants were (ordered from lowest to highest CH_4) *Bergenia crassifolia* root (M4), *Peltiphyllum peltatum* (G1), *S. mahagoni* (I27), *A. villosa* (I2), *Eugenia aquea* (I11), *Myristica fragrans* (I21), *B. crassifolia* leaf (M3), *Pithecellobium jiringa* (I24), *R. typhina* (G2) and *C. hirta* (I8). Screening based on bivariate approach is presented in Figure 1. It appeared that, generally, plants possessed low CH_4 production had low quality, i.e. below the average of total gas production. Among plants that produced low CH_4, only *R. typhina* (G2) resulted in a high total gas production, i.e. 43 ml/200 mg DM. *Salsola laricifolia* (M8), *Hibiscus tiliaceus* (I12) and *Canna indica* (I6) had simultaneously high CH_4 emission and low total gas.

In relation to screening plants based on the multivariate approach, loading and score plots generated from PCA are shown in Figure 2. The PC1 and PC2 explained 42.3% and 23.3% of the

Table 1: Summary of Chemical Composition of Plants from Various Collection Sites and Ruminal Fermentation Variables *in vitro*

Item	CP	EE	NDF	ADF	TP	NTP	TT	CT	HT	Gas	CH_4
	……………………………… (g/kg dry matter) ………………………………									(ml)	(ml/l)
Indonesia (n = 27 species)											
Minimum	79	11	155	135	14	4	2	0	2	5.3	68
Maximum	386	65	710	476	236	102	220	86	206	42.8	185
Mean	215	30	371	281	90	26	65	21	44	23.4	127
SD	90	14	137	100	71	24	66	23	59	10.7	29
Mongolia (n = 14 species)											
Minimum	33	6	226	169	20	9	4	0	0	11.5	44
Maximum	246	35	626	509	320	144	178	175	164	44.8	220
Mean	114	21	444	352	94	43	51	20	33	27.5	153
SD	52	10	136	107	109	47	64	46	51	9.7	43
Switzerland (n = 16 species)											
Minimum	91	6	218	157	8	8	0	0	0	29.5	129
Maximum	247	35	753	344	69	35	38	21	34	49.9	176
Mean	156	19	379	263	31	20	12	3	9	40.6	148
SD	47	8	128	52	15	7	12	6	8	5.8	12
Germany (n = 3 species)											
Minimum	113	17	191	174	57	13	36	1	21	22.2	57
Maximum	169	56	322	185	222	53	209	16	208	49.3	125
Mean	141	31	244	181	160	29	131	11	120	38.2	95
SD	28	22	69	6	90	21	88	8	94	14.2	35
Overall (n = 60 species)											
Minimum	33	6	155	135	8	4	0	0	0	5.3	44
Maximum	386	65	753	509	320	144	220	175	208	49.9	220
Mean	172	25	384	288	79	28	51	15	36	30.0	137
SD	81	13	136	97	79	29	63	28	55	11.9	32

ADF, acid detergent fiber; CP, crude protein; CT, condensed tannins; EE, ether extract; HT, hydrolysable tannins; NDF, neutral detergent fiber; NTP, non-tannin phenols; SD, standard deviation; TP, total phenols; TT, total tannins

total variation, respectively. It is clear that all phenolic fractions were in the opposite direction with CH_4 and total gas production (Figure 2a). However, less clear relationships were observed between the other chemical constituents (CP, EE, NDF and ADF) and the *in vitro* fermentation variables. Some plants were clustered together in reverse direction to that of CH_4 (Figure 2b). These plants were *B. crassifolia* root (M4), *B. crassifolia* leaf (M3), *S. mahagoni* (I27), *C. hirta* (I8), *P. peltatum* (G1), *A. villosa* (I2) and *R. typhina* (G2). Variable CH_4 and total gas production appeared to be in close direction in the loading plot of PCA.

DISCUSSION

Plant Phenolics and CH_4 Emission

Negative relationships between all phenolic fractions and CH_4 emission suggest the role of plant phenolics on mitigating the respective green-house gas under anaerobic condition in the

Table 2. Correlations between Chemical Composition of Plants and Total Gas Production and CH_4 Emission (n = 60)

Variable	Gas	CH_4
CP	0.08	0.05
EE	–0.01	–0.18
NDF	–0.23	0.49***
ADF	–0.27*	0.45***
TP	–0.53***	–0.77***
NTP	–0.35**	–0.52***
TT	–0.51***	–0.73***
CT	–0.46***	–0.33*
HT	–0.36**	–0.67***

ADF, acid detergent fiber; CP, crude protein; CT, condensed tannins; EE, ether extract; HT, hydrolysable tannins; NDF, neutral detergent fiber; NTP, non-tannin phenols; TP, total phenols; TT, total tannins
* P<0.05; ** P<0.01; *** P<0.001

rumen, and this confirms some studies that had been reported previously (Tiemann *et al*., 2008; Bhatta *et al*., 2009). Phenolics, particularly tannins reduce CH_4 emission through several ways, i.e. through reduction in fiber digestion which decreases H_2 production, inhibition of the growth of methanogens (Tavendale *et al*., 2005), and reduction of protozoa population where part of the methanogens are associated symbiotically on the surface or inside the micro-fauna (Vogels *et al*., 1980). Within the tannin classes, based on the correlation data, both CT and HT contributed to lower CH_4 emission as also shown by Bhatta *et al*. (2009). It is hypothesized that lower CH_4 due to CT is more through reduction in fiber digestion as compared to the HT, confirmed by much stronger negative correlation of the former with the total gas production. This might be related to the fact that CT is resistant from microbial degradation in the rumen due to its aromatic structure, while HT is degradable due to the presence of carbohydrate in its structure (McSweeney *et al*., 2001; Makkar, 2003b). Interestingly, the NTP plays also a role in lowering CH_4 with less adverse effect on the total gas production. This is in accordance to our

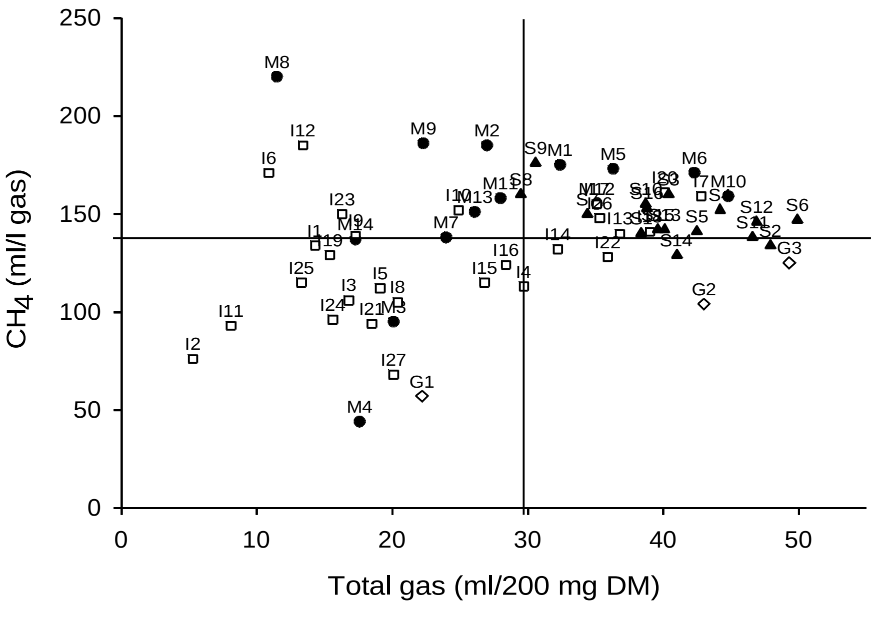
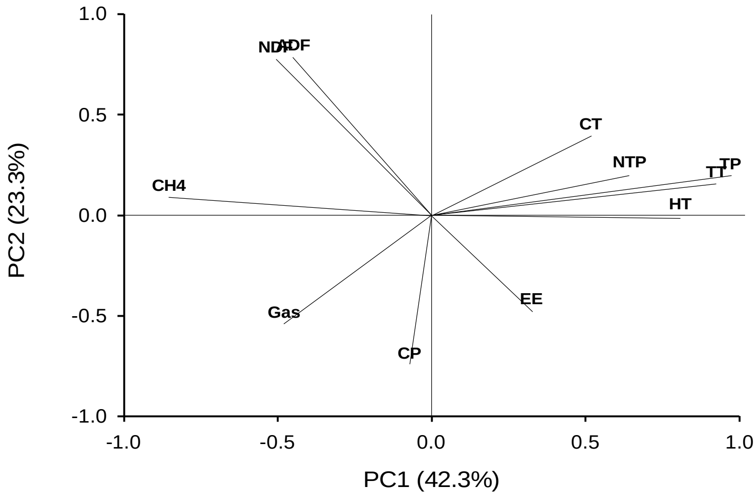
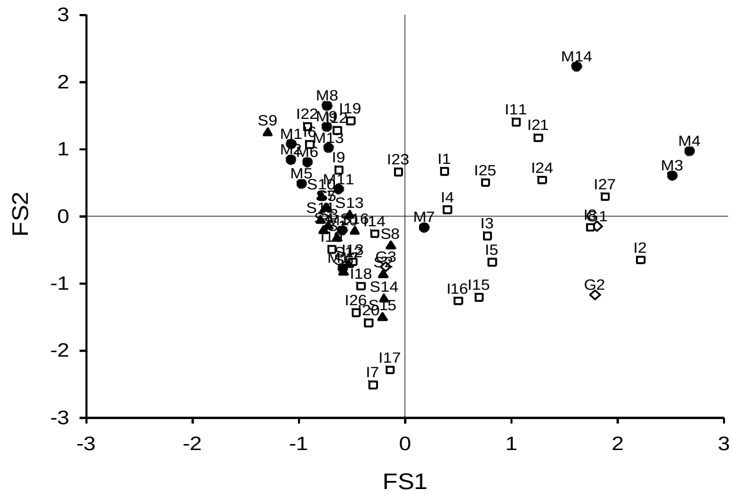


Figure 1: Bivariate approach for screening plants based on total gas produced and CH_4 emission. Vertical and horizontal lines show the average of total gas and CH_4 across all plants, respectively. I, plant samples from Indonesia (□); M, plant samples from Mongolia (●); S, plant samples from Switzerland (▲); G, plant samples from Germany (◊)



(a)



(b)

Figure 2. Loading Plot (a) and Score Plot (b) from Principal Component Analysis as a Multivariate Approach for Screening Plants. ADF, acid detergent fiber; CP, crude protein; CT, condensed tannins; EE, ether extract; FS, factor score; HT, hydrolysable tannins; NDF, neutral detergent fiber; NTP, non-tannin phenols; PC, principal component; TP, total phenols; TT, total tannins; I, plant samples from Indonesia (□); M, plant samples from Mongolia (●); S, plant samples from Switzerland (▲); G, plant samples from Germany (◊)

previous study that the addition of some simple phenolic acids, namely caffeic, p-coumaric, ferulic and cinnamic acids on hay diets at 5 mM concentration decreased *in vitro* CH_4 emission without impairing the organic matter digestibility (Jayanegara *et al*., 2009b).

Different Screening Approaches

Screenings based on univariate, bivariate and multivariate approaches led to similar results to certain extent; the promising plants (low CH_4 production) obtained from univariate approach were also presence when screened by bivariate and multivariate approaches. However, the screening by univariate approach is not as selective as the others. One may obtain plants with low CH_4 production potential, but without considering the other important aspect, i.e. the forage quality. Among the promising plants obtained by the univariate screening, for instance, *A. villosa* (I2) and *E. aquea* (I11) resulted in poor quality performance as shown by the very low total gas production (≤ 10 ml/200 mg DM incubated) of both plants. The bivariate approach may overcome this problem by considering the forage quality related variable (Bodas *et al*., 2008). Although almost all plants possessed low CH_4 emission had low quality, still, a good quality plant could be obtained, i.e. *R. typhina* (G2). What is clear from this approach is to avoid the utilization of plants that possessed high CH_4 emission and low total gas production such as *Salsola laricifolia* (M8), *Canna indica* (I6) and *Hibiscus tiliaceous* (I12).

The loading plot of PCA showed the relationships between variables in a two-dimensional plot. Variables that are in the same direction are positively correlated whereas variables that are in the opposite direction are negatively correlated (Esbensen, 2004). This apparently provides the same message as the correlation analysis, but not only restricted to pairwise comparisons. Since variable CH_4 and total gas production were in a close direction in the loading plot of PCA, it may imply that there is little probability in obtaining many tannin-containing plants with both desired characteristics, i.e. low CH_4 production potential and high forage quality. Therefore, the screening results of this approach were largely in agreement with the univariate approach. This is probably connected to the role of tannins in mitigating CH_4 emission as discussed above but reducing the forage quality as well at the same time. The adverse effects of tannins on forage digestibility have been well-documented (Makkar, 2003b; Mueller-Harvey, 2006). However, this does not exclude the possibility of finding plants with both desired characteristics, like *R. typhina* (G2) in the present dataset, since the structure and the activity of tannins in each plants are quite diverse (Mueller-Harvey, 2006).

CONCLUSION

Plant phenolic contents may be used as an indicator for CH_4 mitigating properties of the plants, although they are also reversely related to the forage quality. Different approaches, i.e. univariate, bivariate and multivariate (PCA) for screening of tannin-containing plants in relation to their ruminal CH_4 emission *in vitro* led to similar results to certain extent. However, screening based on univariate approach has to be conducted cautiously since it does not consider the forage quality, which is also quite an important factor. The bivariate and multivariate approaches may overcome such problem by considering the forage quality related variable. Although almost all tannin-containing plants that possessed low CH_4 emission had low quality in the present study, still, a good quality plant could be obtained, i.e. *R. typhina*. This is possible since great variation have occurred in the structure and activity of each plant phenolics. The searching of plants with both desired characteristics is subjected to further research.

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