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Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis

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ABSTRACT

This study was conducted to elucidate relationships among various phenolic fractions in, and methane (CH_4) emissions from, tropical plants when incubated in ruminal fluid *in vitro*. As a second objective, principal component analysis (PCA) was tested for its utility in screening plants for their ability to reduce CH_4 formation at simultaneously acceptable nutritional quality. Leaves from 27 tropical plants were analyzed for their nutritional composition and various phenolic fractions. They were incubated *in vitro* using the Hohenheim gas test method. Variables measured after 24 h of incubation were total gas and CH_4 production, and pH, ammonia, bacterial and protozoal counts, as well as short-chain fatty acids in the incubation fluid. *In vitro* organic matter (OM) digestibility was computed by a standard equation. The data obtained was subjected to analysis of variance, correlation, regression and PCA. Among phenolic fractions, total phenols had the closest relationship with CH_4/digestible OM ($r = -0.84$, $P<0.001$). The total tannin fraction contributed strongly to this effect ($r = -0.74$, $P<0.001$) whereas the non-tannin phenol fraction was less important ($r = -0.45$, $P<0.05$). Methane reduction by the influence of non-tannin phenols was not associated with a negative effect on protein degradation, while this was the case with tannins. Condensed ($r = -0.60$, $P<0.01$) and hydrolysable tannins ($r = -0.60$, $P<0.01$) contributed to the decrease in CH_4/digestible OM. The loading plot of PCA showed that dietary crude protein (CP) content and incubation fluid ammonia, total short-chain fatty acids, propionate, valerate, *iso*-butyrate, *iso*-valerate as well as *in vitro* OM digestibility were clustered. They had inverse directions to contents of fiber fractions and incubation fluid acetate proportion and acetate-to-propionate ratio. The methane-to-total-gas ratio had the opposite effect of the contents of any phenolic fraction. Plants possessing a favorable forage quality, based on the corresponding PCA score plot, were *Carica papaya*, *Manihot esculenta*, *Morinda citrifolia*, *Sesbania grandiflora* and *Melia azedarach*, whereas CH_4 mitigating plants included *Swietenia mahagoni*, *Acacia villosa*, *Eugenia aquea*, *Myristica fragrans* and *Clidemia hirta*. All phenolic fractions studied reduced CH_4 emissions from *in vitro* incubations with ruminal fluid and PCA seems useful to screen plants for high nutritional quality and low ruminal CH_4 formation. However, high forage quality seemed to be partially associated with high

Abbreviations: ADFom, acid detergent fiber; aNDFom, neutral detergent fiber; CP, crude protein; CT, condensed tannins; DM, dry matter; EE, ether extract; HGT, Hohenheim gas test; HT, hydrolysable tannins; IVOMD, *in vitro* OM digestibility; lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid; MSD, minimum significant difference; NTP, non-tannin phenols; OM, organic matter; PC, principal component; PCA, principal component analysis; PSM, plant secondary metabolites; SCFA, short-chain fatty acids; TP, total phenols; TT, total tannins.

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CH_4 emission. The search for plants rich in non-tannin phenols might be promising as these compounds appear to decrease CH_4 while they obviously have less negative effect on protein degradation as compared to the tannin fractions.

1. Introduction

Methane is among the most important greenhouse gases and its global atmospheric concentration has substantially increased in the last 150 years where, after a few years at a plateau, it seems to be increasing again (Mascarelli, 2009). Livestock, especially ruminants, contribute to CH_4 emissions through enteric fermentation and processes taking place in the manure (IPCC, 2006). It was estimated that, globally, ruminant livestock produce 80 million tonnes of CH_4 annually which accounts for 0.28 of total anthropogenic methane emissions (Beauchemin et al., 2008) which itself makes up 0.63 of total global methane emissions (EPA, 2010). Moreover, CH_4 from enteric fermentation also represents a loss of energy to the ruminant of up to 0.12 of gross energy intake (McCrabb and Hunter, 1999). This is especially the case when feeding highly fibrous diets prevalent in the tropics.

Inclusion of feeds containing plant secondary metabolites (PSM) such as saponins or phenols (especially tannins) in diets seems promising as a nutritional strategy for the tropics to reduce CH_4 emissions from ruminants (Puchala et al., 2005; Goel et al., 2008) because many tropical plants are rich in PSM. Such an abundance of PSM in the plant has been explained as a response to environmental stress and is assumed to promote resistance against stress and robustness when mechanically or environmentally damaged (Vogt, 2010). Up to now, the effect of phenols on ruminal CH_4 production was mainly investigated by focusing on tannins and, within this group, on condensed tannins (CT; Carulla et al., 2005; Puchala et al., 2005; Tiemann et al., 2008; Grainger et al., 2009). Research on effects of hydrolysable tannins (HT) has attracted less interest and the effects seem to be inconsistent. Addition of a small amount of HT from chestnut wood extract did not decrease CH_4 emissions from lambs in the study of Śliwiński et al. (2002). However, Bhatta et al. (2009) reported that adding HT extracts from myrabolam and chestnut reduced CH_4 emissions *in vitro* to some degree, and that the effects were higher when HT were combined with CT. Even less attention has been given to potential effects of non-tannin phenols (NTP) on ruminal fermentation and methanogenesis (Evans and Martin, 2000; Jayanegara et al., 2009a).

Extensive screenings of plants and plant extracts that have CH_4 reducing properties have been conducted (Bodas et al., 2008; Garcia-Gonzalez et al., 2008; Soliva et al., 2008). Screening methods are mainly performed based on univariate or bivariate approaches although many variables in the plants and their extracts need to be considered including proximate composition, cell wall constituents, PSM profile, fermentation products, digestibility and CH_4 emission itself. This suggests that it may be useful to apply a multivariate statistical approach to those variables. Principal component analysis (PCA) is such a method which allows identification of the most important directions to explain the variation in a multivariate data matrix in a graphical plot. The PCA reduces the dimensionality of data, transforms the original variables into new axes or principal components (PC) and retains as much as possible of the total variation in the data in only a few PC (Härdle and Simar, 2007). Since many variables in the plant and in the rumen may be related to ruminal CH_4 production and forage quality, assessing their relationships by PCA by screening plants for low ruminal CH_4 production potential and high nutritional quality seems promising.

A first objective was to elucidate relationships among various phenolic fractions, a differentiation rarely made in CH_4 research. Total phenols (TP), total tannins (TT), CT, HT, and NTP were distinguished. Production of CH_4 was studied *in vitro* by incubating various tropical plants containing these phenol fractions in different concentrations in ruminal fluid. Secondly, we aimed to test the utility of PCA for screening of forage plants that have a low CH_4 mitigating potential and simultaneously an acceptable forage quality.

2. Materials and methods

2.1. Experimental plants

Samples from 27 plant species were collected in November 2008 from two sites of the area of Bogor, West Java, Indonesia (Table 1). The first site was located at Bogor Agricultural University, Darmaga, at 180 m elevation and the second was situated at the Indonesian Research Institute for Animal Production, Ciawi, at 350 m elevation. Samples included above ground biomass of one grass species and four herb species as well as leaves of nine shrub species and 13 trees species. Only species were considered which are either common in ruminant feeding in rural areas or are traditional veterinary medicinal plants in Indonesia. Approximately 3 kg wet weight of each plant was collected, and each sample consisted of several individual plants from the same species. After collection, plant samples were immediately stored indoors for 2 days, followed by oven drying at 50 °C and grinding to pass a 1 mm sieve prior to chemical analysis and *in vitro* incubation.

Table 1
Chemical composition of the experimental plants (g/kg dry matter; $n_{replicate} = 2$).

No.	Plant species	Plant family	Sample type	Organic matter	Crude protein	Ether extract	aNDFom[a]	ADFom[b]	Lignin (sa)	Non-fiber CH[c]
1	*Acacia mangium*	Fabaceae	Shrub leaves	939	162	33	436	349	198	203
2	*Acacia villosa*	Fabaceae	Shrub leaves	949	311	27	246	148	80	129
3	*Albizia falcataria*	Fabaceae	Tree leaves	926	223	39	310	223	111	241
4	*Artocarpus heterophyllus*	Moraceae	Tree leaves	857	142	27	339	275	64	253
5	*Calliandra calothyrsus*	Fabaceae	Shrub leaves	924	286	34	326	184	69	151
6	*Canna indica*	Cannaceae	Herb	857	158	13	581	375	106	91
7	*Carica papaya*	Caricaceae	Tree leaves	905	386	47	155	135	46	292
8	*Clidemia hirta*	Melastomataceae	Shrub leaves	927	129	22	232	181	69	328
9	*Cycas rumphii*	Cycadaceae	Tree leaves	886	209	13	509	386	131	129
10	*Erythrina orientalis*	Fabaceae	Tree leaves	913	343	15	447	272	100	88
11	*Eugenia aquea*	Myrtaceae	Tree leaves	941	100	35	479	382	181	158
12	*Hibiscus tiliaceus*	Malvaceae	Shrub leaves	934	168	16	541	438	156	154
13	*Ipomoea batatas*	Convolvulaceae	Herb	904	236	22	334	273	85	283
14	*Lantana camara*	Verbenaceae	Herb	916	186	28	382	296	130	273
15	*Leucaena diversifolia*	Fabaceae	Shrub leaves	930	336	33	249	147	90	198
16	*Leucaena leucocephala*	Fabaceae	Shrub leaves	904	306	41	263	185	94	198
17	*Manihot esculenta*	Euphorbiaceae	Shrub leaves	915	377	65	211	186	96	230
18	*Melia azedarach*	Meliaceae	Tree leaves	895	253	19	253	223	105	340
19	*Mimosa invisa*	Fabaceae	Herb	918	152	11	555	476	198	155
20	*Morinda citrifolia*	Rubiaceae	Tree leaves	880	228	56	223	219	86	348
21	*Myristica fragrans*	Myristicaceae	Tree leaves	950	101	20	380	361	196	268
22	*Paspalum dilatatum*	Poaceae	Grass	925	79	13	710	424	52	98
23	*Persea americana*	Lauraceae	Tree leaves	940	149	38	480	355	188	200
24	*Pithecellobium jiringa*	Fabaceae	Tree leaves	963	215	37	472	389	207	46
25	*Psidium guajava*	Myrtaceae	Tree leaves	921	146	35	385	294	120	223
26	*Sesbania grandiflora*	Fabaceae	Shrub leaves	895	312	28	251	200	70	285
27	*Swietenia mahagoni*	Meliaceae	Tree leaves	899	112	45	281	222	78	254

[a] Neutral detergent fiber.
[b] Acid detergent fiber.
[c] Non-fiber carbohydrates = (organic matter – crude protein – ether extract – aNDFom – total phenols).

2.2. Analysis of chemical composition

Plants were analyzed by standard procedures of AOAC (1997) and Van Soest et al. (1991). Contents of dry matter (DM) and total ash were determined with a TGA-500 furnace (Leco Corporation, St. Joseph, MI, USA; AOAC no. 942.05) heated at steps of 105 °C and 550 °C, respectively. A C/N analyzer (Leco-Analysator Typ FP-2000, Leco Instrumente GmbH, Kirchheim, Germany) was used to determine N (AOAC no. 977.02). Ether extract (EE) was analyzed with a Soxhlet extractor (Extraktionssystem B-811, Büchi, Flawil, Switzerland; AOAC no. 963.15). Neutral detergent fiber (aNDFom, with α-amylase and without sodium sulfite), acid detergent fiber (ADFom) and lignin(sa) were determined according to Van Soest et al. (1991) using the Fibertec apparatus (Fibertec System M, Tecator, 1020 Hot Extraction, Flawil, Switzerland). Fiber values are expressed without residual ash. Non-fiber carbohydrates were calculated by subtracting CP, EE, aNDFom and TP from organic matter (OM).

Determinations of TP, TT and CT were completed based on Makkar (2003a). For the extraction step, approximately 100 mg of ground plant sample (ground through a 1.0 mm screen) was weighed and inserted in a 15 ml plastic tube. Five ml of aqueous acetone (700:300 v/v) was added to the sample, vortexed and subjected to ultrasonic treatment for 20 min at room temperature (*i.e.*, 25 °C) in an ultrasonic water bath (TEC-25, Telsonic AG, Bronschhofen, Switzerland). Subsequently, the tube was centrifuged for 10 min at 3000 × *g* at 4 °C (Omnifuge 2.0 RS, ThermoFisher Scientific, Waltham, MA, USA) and the supernatant was collected. This procedure was repeated twice and the two supernatants were combined. The TP and TT were then determined by a modified Folin-Ciocalteu method using polyvinyl-polypyrrolidone (PVPP) to separate tannin phenols from NTP. Between 0.02 and 0.1 ml of the extract (depending on the concentration of TP and TT in the plant sample) was put in a test tube and filled up to the volume of 0.5 ml with distilled water. Folin-Ciocalteu reagent (0.25 ml; 1 N; Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and 1.25 ml sodium carbonate solution (40 g $Na_2CO_3 \cdot 10H_2O$ in 200 ml distilled water) were added. The tube was vortexed and absorbance for TP was recorded at 725 nm after 40 min using a UV–vis spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). For measuring TT, 100 mg PVPP (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) in a test tube was added by 1.0 ml distilled water and 1.0 ml of the extract. The mixture was vortexed and kept at 4 °C for 15 min, centrifuged (3000 × *g*, 4 °C, 10 min), and the supernatant, containing only the NTP, was decanted. Measurement of the phenolic content of the supernatant was as described above. Both TP and TT were calibrated against gallic acid solution as a standard (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and values were expressed as gallic acid equivalents. Condensed tannins were determined by the butanol–HCl–iron method. A total of 0.25 ml of the extract was mixed with aqueous acetone (700:300 v/v), was pipetted in a glass tube. The ratio of extract to acetone depended on CT concentration in the sample. Fifty microliter of ferric reagent (2.0 g ferric ammonium sulfate in 100 ml of 2 N HCl) was added to the tube followed by 1.5 ml butanol–HCl (950:50 v/v). The tube was closed, vortexed and heated in a boiling water bath at 100 °C for 60 min. After cooling, the absorbance was read at 550 nm using the same UV–vis spectrophotometer as for TP and TT and the value from a blank (*i.e.*, absorbance of the unheated mixture) was subtracted. The CT values obtained in the plant samples were expressed as leucocyanidin equivalents. The HT were estimated as the difference between TT and CT (Singh et al., 2005). Chemical analyses were in duplicate or triplicate (detergent fractions only).

2.3. In vitro incubation

In vitro incubation used the Hohenheim gas test (HGT) apparatus according to Menke and Steingass (1988), with modified syringes as described by Soliva and Hess (2007). The plant samples were incubated at 39 °C for 24 h in four replicates (runs) each and comprised two syringes for each replicate. In each syringe, 200 mg DM of plant sample was incubated with 10 ml of ruminal fluid and 20 ml of HGT buffer solution (Menke et al., 1979). Ruminal fluid was obtained before the morning feeding from a rumen fistulated lactating Brown Swiss cow, which was housed according to Swiss guidelines for animal welfare. The cow was fed white clover/ryegrass hay (*ad libitum*) and 0.5 kg/day of a concentrate for dairy cattle (UFA 149, UFA AG, Herzogenbuchsee, Switzerland) containing, per kg of DM, 7.35 MJ net energy for lactation and 390 g crude protein (CP). Ruminal fluid was strained through four layers of gauze (1 mm pore size, Type 17 MedPro, Novamed AG, Flawil, Switzerland) before use. Additionally, syringes without feed (blanks), with standard concentrate and with standard hay (obtained from the Institute for Animal Nutrition, University of Hohenheim, Stuttgart, Germany) were incubated in triplicate in each run as recommended by Menke and Steingass (1988), serving as control for the incubation and for calculation of *in vitro* OM digestibility (IVOMD; see below).

2.4. Post fermentation analyses and calculations

After 24 h of incubation, fermentation gas volume was read from the calibrated scale on the glass syringes. The incubation was terminated by decanting the liquid phase from all syringes while the fermentation gas remained inside. Subsequently, 0.15 ml of fermentation gas was withdrawn from the incubation unit with a Hamilton syringe (Hamilton, AG, Bonaduz, Switzerland) through a gas-tight septum covering the second outlet of the incubation syringe. These samples were analyzed on a gas chromatograph (model 5890 Series II, Hewlett Packard, Avondale, PA, USA) for CH_4 concentration as described by Soliva and Hess (2007).

Samples of the incubation fluid were analyzed for pH and ammonia with a potentiometer (model 632, Metrohm, Herisau, Switzerland) equipped with the respective electrodes. Total protozoal and bacterial counts in the samples were determined by direct microscopic counting using 0.1 mm and 0.02 mm depth Bürker counting chambers (Blau Brand, Wertheim,

Germany), respectively. Prior to bacterial counting, samples were fixed with Hayem solution ($HgCl_2$, 2.5 mg/ml; Na_2SO_4, 25 mg/ml; NaCl, 5.0 mg/ml). For protozoal counting, samples were treated with 1:10 diluted formalin (400/1000 w/v in water). Short chain fatty acids (SCFA) in the incubation fluid were analyzed on a HPLC (LaChrom, L-7000 series, Hitachi Ltd., Tokyo, Japan) with an UV VIS detector at 210 nm by the method of Ehrlich et al. (1981).

Following the equations (Menke and Steingass, 1988), IVOMD (mg/g) was calculated as:

$$148.8 + 8.893\,\text{gas production (ml)} + 0.448\,\text{CP (g/kg DM)} + 0.651\,\text{total ash (g/kg DM)}$$

where the gas production value obtained after 24 h incubation period was corrected for the values with the different controls thus accounting for differences in composition and activity of the ruminal fluid among runs. At first, gas production from the blank was subtracted from all plant samples and other controls incubated to obtain the net gas production. In order to correct for the values obtained with the hay and concentrate standards, gas production from the hay standard (44.43 ml gas/200 mg DM; 24 h incubation) was divided by the measured net value for the hay standard to provide the correction factor F_H. Similarly, the gas production from concentrate standard (65.18 ml/200 mg DM; 24 h incubation) was divided by its measured net gas production to provide the correction factor F_C. The mean of these two correction factors was used for correction of the measurements made in the samples following Menke and Steingass (1988).

2.5. Statistical analysis

Data was subjected to analysis of variance using procedure GLM of SAS (2008). The model used was based on a randomized complete block design using the following model:

$$Y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$$

where Y_{ij} is the observed value for the jth replicate of the ith treatment, μ is the grand mean, τ_i is the treatment effect ($n = 27$ plants) for the ith treatment (fixed effect), β_j is the block effect (replicate) for the jth block (fixed effect), and ε_{ij} is the random error associated with the Y_{ij} experimental unit.

Following general linear model (GLM), the minimum significant difference (MSD) was generated from Tukey's test as the basis of the multiple comparisons among means. Pearson correlation coefficients between variables, and their significance, were computed by the procedure CORR, and linear regression equations were generated by the procedure REG of SAS. Principal component analysis was performed using the procedure FACTOR of SAS (method = prin) with Kaiser's criterion *i.e.*, Eigenvalue ≥ 1.0 (mineigen = 1; Kaiser, 1960) to extract the principal components. No rotation method was applied to the principal components. This procedure generated loading and score vectors, where loading vectors are the correlations between the variables and the extracted principal components (or 'new' variables) and score vectors are the scores of each individual case (or experimental plant in the present study) on each principal component. Only the first two principal components were plotted, both for loading and score plots, since they represented the majority of total variation. The loading plot is used for describing the relationship among variables investigated and the score plot is used for classifying the experimental plants according to the loading vectors generated. All variables were standardized by a mean of zero and a variance of one prior to PCA. All figures were generated using SigmaPlot (2008).

3. Results

3.1. Plant chemical composition

There were some plants with ash contents of >100 g/kg DM (Table 1). The CP contents varied among plants from 79 (in *Paspalum dilatatum*) to 386 g/kg DM (in *Carica papaya*). There were other plants containing CP > 300 g/kg DM (*i.e.*, *Acacia villosa*, *Erythrina orientalis*, *Leucaena diversifolia*, *Leucaena leucocephala*, *Manihot esculenta* and *Sesbania grandiflora*). Most plants had only moderate EE contents, except *M. esculenta* and *Morinda citrifolia* which contained >50 g EE/kg DM. Cell wall contents varied widely among plants, ranging from 155 to 710, 135 to 476 and 46 to 207 g/kg DM for aNDFom, ADFom and lignin(sa), respectively. Plants rich in CP (*i.e.*, >300 g/kg DM) had relatively low cell wall contents. This was especially true for *C. papaya*. There was also an inverse relationship between contents of fiber and non-fiber carbohydrates, presumably mainly starch and sugars. The latter ranged from values as low as 46 (*Pithecellobium jiringa*) to values as high as 348 g/kg DM (*M. citrifolia*).

Contents of TP were highest in *A. villosa* (236 g/kg DM) and lowest in *Canna indica* (14 g/kg DM; Table 2). The NTP and TT ranged from 4 (*Clidemia hirta*) to 102 g/kg DM (*Eugenia aquea*) and from 2 (*S. grandiflora*) to 220 g/kg DM (*A. villosa*), respectively. There were three plants (*C. papaya*, *Ipomoea batatas* and *S. grandiflora*) where no CT was detected. Plants rich in HT (>100 g/kg DM) included *A. villosa*, *C. hirta*, *Myristica fragrans* and *P. jiringa*.

3.2. Effects of plant species on in vitro fermentation characteristics

The plant species effect was prominent (P<0.001) for all fermentation variables (Tables 3 and 4). The highest incubation fluid NH_3 concentration was with *M. esculenta* (24.8 mmol/l, equivalent to 3.72 mmol/g DM of the material incubated) and

Table 2
Content of phenolic fractions of the experimental plants (g/kg dry matter; $n_{replicate} = 2$).

No.	Plant species	Total phenols	Non-tannin phenols	Total tannins	Condensed tannins	Hydrolysable tannins
1	*Acacia mangium*	105	14	91	42	49
2	*Acacia villosa*	236	16	220	14	206
3	*Albizia falcataria*	113	49	64	46	18
4	*Artocarpus heterophyllus*	96	16	80	47	33
5	*Calliandra calothyrsus*	127	46	81	22	59
6	*Canna indica*	14	6	8	2	6
7	*Carica papaya*	25	17	8	0	8
8	*Clidemia hirta*	216	4	212	10	202
9	*Cycas rumphii*	26	7	19	11	8
10	*Erythrina orientalis*	20	16	4	1	3
11	*Eugenia aquea*	169	102	67	40	27
12	*Hibiscus tiliaceus*	55	13	42	23	19
13	*Ipomoea batatas*	29	13	16	0	16
14	*Lantana camara*	47	28	19	2	17
15	*Leucaena diversifolia*	114	56	58	34	24
16	*Leucaena leucocephala*	96	29	67	18	49
17	*Manihot esculenta*	32	16	16	4	12
18	*Melia azedarach*	30	17	13	4	9
19	*Mimosa invisa*	45	15	30	17	13
20	*Morinda citrifolia*	25	15	10	3	7
21	*Myristica fragrans*	181	6	175	72	103
22	*Paspalum dilatatum*	25	11	14	1	13
23	*Persea americana*	73	27	46	34	12
24	*Pithecellobium jiringa*	193	8	185	10	175
25	*Psidium guajava*	132	62	70	34	36
26	*Sesbania grandiflora*	19	17	2	0	2
27	*Swietenia mahagoni*	207	69	138	86	52

Table 3
pH, ammonia and short chain fatty acid profiles when incubating the experimental plants ($n_{replicate} = 8$).

No.	Plant species	pH	NH_3 (mmol/l)	Short chain fatty acids (molar proportion)[a]							
				Total (mmol/l)	C_2	C_3	C_4	*iso* C_4	C_5	*iso* C_5	C_2/C_3
1	*Acacia mangium*	7.48	10.8	45.2	74.2	17.3	7.0	0.61	0.39	0.46	4.29
2	*Acacia villosa*	7.49	11.5	42.4	71.3	18.8	7.8	0.96	0.69	0.54	3.83
3	*Albizia falcataria*	7.44	11.9	58.4	76.7	15.4	6.4	0.70	0.38	0.44	5.03
4	*Artocarpus heterophyllus*	7.42	9.5	61.3	74.9	16.9	6.4	0.77	0.39	0.60	4.47
5	*Calliandra calothyrsus*	7.39	9.7	54.0	76.4	15.8	6.0	0.76	0.45	0.60	4.95
6	*Canna indica*	7.57	14.7	56.9	73.5	16.5	8.1	0.94	0.44	0.58	4.49
7	*Carica papaya*	7.30	22.3	79.2	69.1	19.3	7.4	1.67	0.81	1.72	3.62
8	*Clidemia hirta*	7.37	8.7	53.0	75.3	15.5	7.5	0.79	0.42	0.54	4.93
9	*Cycas rumphii*	7.42	14.5	53.6	74.4	15.7	7.9	0.73	0.47	0.82	4.77
10	*Erythrina orientalis*	7.59	22.2	60.2	71.9	17.6	7.2	1.59	0.57	1.13	4.10
11	*Eugenia aquea*	7.44	10.3	42.9	76.1	14.5	8.0	0.61	0.40	0.39	5.31
12	*Hibiscus tiliaceus*	7.55	10.8	50.6	74.7	15.4	7.9	1.06	0.41	0.52	4.87
13	*Ipomoea batatas*	7.30	18.0	71.7	74.3	16.5	7.3	0.92	0.40	0.57	4.52
14	*Lantana camara*	7.42	12.1	63.6	73.8	17.3	7.2	0.74	0.50	0.59	4.37
15	*Leucaena diversifolia*	7.42	15.4	60.8	74.5	17.9	5.6	0.85	0.40	0.72	4.21
16	*Leucaena leucocephala*	7.41	16.3	63.6	74.0	18.5	6.1	0.63	0.46	0.40	4.01
17	*Manihot esculenta*	7.41	24.8	75.9	71.9	17.4	7.3	1.40	0.75	1.30	4.17
18	*Melia azedarach*	7.29	18.2	72.5	71.7	19.5	6.7	0.85	0.53	0.74	3.79
19	*Mimosa invisa*	7.53	11.2	55.7	73.2	18.2	6.5	1.05	0.61	0.53	4.08
20	*Morinda citrifolia*	7.31	19.5	75.2	69.9	21.2	6.5	1.30	0.44	0.65	3.36
21	*Myristica fragrans*	7.34	11.6	53.6	76.3	14.5	7.2	0.77	0.44	0.79	5.34
22	*Paspalum dilatatum*	7.49	8.9	62.2	73.1	16.8	7.7	1.36	0.51	0.58	4.38
23	*Persea americana*	7.40	12.8	56.6	75.9	14.8	7.8	0.75	0.36	0.44	5.15
24	*Pithecellobium jiringa*	7.53	16.2	56.6	75.5	15.6	7.0	0.68	0.62	0.58	5.00
25	*Psidium guajava*	7.32	11.6	49.6	74.1	16.3	8.0	0.78	0.36	0.44	4.65
26	*Sesbania grandiflora*	7.50	21.5	74.6	72.7	18.7	6.0	1.43	0.54	0.67	3.92
27	*Swietenia mahagoni*	7.37	7.6	50.8	75.8	16.7	5.4	0.81	0.48	0.75	4.55
	SEM	0.012	0.48	0.95	0.18	0.16	0.08	0.037	0.013	0.026	0.050
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	MSD[b]	0.127	5.20	10.45	3.16	2.94	1.35	0.870	0.290	0.493	0.870

[a] C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_2/C_3, acetate/propinate ratio, no unit.
[b] Minimum significant difference.

Table 4
Total gas, CH_4 production, digestibility[a], bacterial and protozoal counts when incubating the experimental plants ($n_{replicate}$ = 8).

No.	Plant species	Total gas (ml)	CH_4 (ml)	CH_4/total gas (ml/l)	IVOMD (mg/g)	Bacteria (10^9/ml)	Protozoa (10^4/ml)
1	*Acacia mangium*	14.3	2.63	134	408	4.87	2.97
2	*Acacia villosa*	5.3	0.84	76	373	4.60	2.45
3	*Albizia falcataria*	16.8	2.39	106	466	4.11	3.00
4	*Artocarpus heterophyllus*	29.7	3.70	113	592	4.68	1.95
5	*Calliandra calothyrsus*	19.1	2.34	112	512	3.79	1.72
6	*Canna indica*	10.9	2.70	171	420	2.52	1.06
7	*Carica papaya*	42.8	7.36	159	825	3.39	2.39
8	*Clidemia hirta*	20.4	2.68	105	466	3.12	1.72
9	*Cycas rumphii*	17.3	3.03	139	495	2.85	1.53
10	*Erythrina orientalis*	24.9	4.53	152	613	3.14	2.00
11	*Eugenia aquea*	8.1	1.03	93	303	4.03	1.53
12	*Hibiscus tiliaceus*	13.4	2.79	185	398	3.36	2.33
13	*Ipomoea batatas*	36.8	5.54	140	695	3.62	3.00
14	*Lantana camara*	32.2	4.90	132	615	3.10	2.00
15	*Leucaena diversifolia*	26.8	3.71	115	607	3.29	2.25
16	*Leucaena leucocephala*	28.4	4.15	124	644	3.87	2.11
17	*Manihot esculenta*	35.1	6.22	155	723	4.13	1.75
18	*Melia azedarach*	39.0	5.86	141	713	3.01	2.47
19	*Mimosa invisa*	15.4	2.40	129	423	2.49	1.97
20	*Morinda citrifolia*	40.1	7.21	161	747	4.01	1.53
21	*Myristica fragrans*	18.5	1.99	94	408	4.26	2.78
22	*Paspalum dilatatum*	35.9	5.03	128	587	3.83	2.06
23	*Persea americana*	16.3	3.18	150	418	3.18	2.00
24	*Pithecellobium jiringa*	15.6	2.05	96	427	4.70	1.81
25	*Psidium guajava*	13.3	2.11	115	399	3.65	2.11
26	*Sesbania grandiflora*	35.3	5.97	148	724	3.46	2.14
27	*Swietenia mahagoni*	20.1	1.59	68	458	5.79	1.72
	SEM	0.74	0.136	2.2	9.6	0.109	0.065
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	MSD[b]	4.43	0.760	16.9	45.0	1.948	1.315

[a] IVOMD, *in vitro* organic matter digestibility.
[b] Minimum significant difference.

lowest with *Swietenia mahagoni* (7.6 mmol/l). Fermentation of *C. papaya* resulted in the highest total incubation fluid SCFA concentration and almost the lowest C_2/C_3 ratio among all plants. Total SCFA with *C. papaya* differed (P<0.05 based on MSD) from all other plants except *M. esculenta*, *M. citrifolia*, *S. grandiflora*, *Melia azedarach* and *I. batatas*.

The pattern of total gas production (Table 4) was closely related to that of total SCFA concentration (Table 3). This was also the case for IVOMD since this value was partially derived from total gas production. *Carica papaya* produced the most total gas, and was also highest in IVOMD among all plants. The next ranked plants were *M. citrifolia*, *M. azedarach*, *I. batatas*, *P. dilatatum*, *S. grandiflora* and *M. esculenta*. When searching for low CH_4 production, the absolute CH_4 amount is less indicative than the CH_4 proportion of total gas or in relation to digestible OM. The CH_4/total gas ratio was lowest with *S. mahagoni* (68 ml/l). Other plants, which resulted in <100 ml CH_4/liter of total gas, were *A. villosa*, *E. aquea*, *M. fragrans* and *P. jiringa*. The bacterial counts ranged from 2.5 (*Mimosa invisa*) to 5.8×10^9/ml (*S. mahagoni*) and the protozoal counts covered a range from 1.1 (*C. indica*) to 3.0×10^4/ml (*Albizia falcataria* and *I. batatas*).

3.3. Relationships between plant compositional values and fermentation variables

There were close relationships between dietary CP contents and various fermentation traits, the most prominent being that with ammonia concentration, whereas correlations were rarely significant in the case of EE (Table 5). High contents of non-fiber carbohydrates were associated with a low pH and a low CH_4/total gas ratio. Cell wall fractions did not have a close relationship with CH_4/digestible OM, whereas this was the case with all phenolic fractions (*i.e.*, negative and linear; Fig. 1). Among phenolic fractions, TP had the closest relationship ($r = -0.84$, P<0.001). This relationship effect mainly resulted from the TT fraction ($r = -0.74$, P<0.001) and less so from the NTP fraction ($r = -0.45$, P<0.05). The regression slopes indicate that the NTP fraction was more effective in reducing CH_4/digestible OM than TT. The CT and the HT fractions both contributed (P<0.01) to the decrease of CH_4/digestible OM (both $r = -0.60$). Variation among plants in CH_4/digestible OM decreased with increasing concentration of any of the phenolic fractions.

3.4. Results of the principal component analysis

As a result of the PCA, 58.2% of the total variation was explained by the first two principal components (37.0% and 21.2%, respectively; Fig. 2). The position of each variable in the loading plot (Fig. 2a) describes its relationship to the other variables. Variables which are close have high correlations. Variables on the same side of the origin (0,0) are positively correlated

Table 5
Pearson correlation coefficients between plant composition and *in vitro* incubation fluid parameters (n_{plant} = 27).

	Crude protein	Ether extract	aNDFom	ADFom	Lignin (sa)	Non-fiber carbohydrates	Total phenols	Non-tannin phenols	Total tannins	Condensed tannins	Hydrolysable tannins
pH	−0.04	−0.41*	0.59**	0.46*	0.27	−0.66***	−0.08	−0.18	−0.02	−0.16	0.04
NH_3	0.78***	0.33	−0.40*	−0.36	−0.21	−0.25	−0.57**	−0.30	−0.50**	−0.58**	−0.34
Total SCFA	0.54**	0.32	−0.43*	−0.39*	−0.47*	−0.06	−0.66***	−0.34	−0.59**	−0.53**	−0.46*
C_2	−0.55**	−0.16	0.30	0.32	0.41*	0.18	0.50**	0.38	0.40*	0.63***	0.20
C_3	0.55**	0.25	−0.47*	−0.47*	−0.44*	0.01	−0.39*	−0.23	−0.33	−0.45*	−0.20
C_4	−0.27	−0.30	0.47*	0.45*	0.26	−0.30	−0.09	−0.21	−0.02	−0.31	0.09
iso C_4	0.49**	0.09	−0.12	−0.20	−0.46*	−0.33	−0.55**	−0.33	−0.47*	−0.52**	−0.32
C_5	0.59**	0.22	−0.26	−0.25	−0.20	−0.17	−0.13	−0.31	−0.03	−0.44*	0.13
iso C_5	0.59**	0.28	−0.37	−0.36	−0.35	−0.11	−0.33	−0.23	−0.28	−0.25	−0.21
C_2/C_3	−0.58**	−0.20	0.42*	0.45*	0.48*	0.08	0.45*	0.28	0.38	0.50**	0.23
Total gas	0.41*	0.27	−0.42*	−0.41*	−0.56**	0.06	−0.58**	−0.27	−0.52**	−0.43*	−0.42*
CH_4	0.51**	0.27	−0.36	−0.34	−0.47*	−0.12	−0.74***	−0.36	−0.66***	−0.60***	−0.51**
CH_4/tot. gas	0.28	−0.08	0.17	0.17	−0.05	−0.55**	−0.87***	−0.46*	−0.77***	−0.60***	−0.62***
IVOMD	0.45*	0.28	−0.45*	−0.43*	−0.56**	−0.02	−0.59**	−0.28	−0.53**	−0.46*	−0.41*
Bacteria	−0.13	0.49**	−0.25	−0.20	−0.02	0.43*	0.59**	0.27	0.53**	0.60***	0.36
Protozoa	0.13	−0.06	−0.21	−0.13	0.10	0.27	0.06	−0.11	0.11	0.20	0.04

aNDFom, neutral detergent fiber; ADFom, acid detergent fiber; SCFA, short-chain fatty acid; C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_2/C_3, acetate/propinate ratio, no unit; IVOMD, *in vitro* organic matter digestibility.
* P<0.05.
** P<0.01.
*** P<0.001.

and those on the opposite side of origin are negatively correlated. Accordingly, dietary CP and incubation fluid NH_3, total SCFA, IVOMD, C_3, C_5, *iso* C_4 and *iso* C_5 were clustered, and oriented in an inverse direction to a cluster composed of dietary aNDFom, ADFom and lignin(sa), and incubation fluid C_2, C_2/C_3. The CH_4 proportion of total gas had an opposite direction to all phenolic fractions (*i.e.*, TP, NTP, TT, CT and HT). This is consistent with the regression analysis (Fig. 1). The individual experimental plants were then classified based on the loading plot result, combined with the factor score of each plant (Fig. 2b). Plants which were arranged in the same direction with clustered variables of dietary CP, incubation fluid NH_3, total SCFA, IVOMD, C_3, C_5, *iso* C_4 and *iso* C_5 were considered to have good quality, and *vice versa*. Similarly, plants which were in the same direction with CH_4/total gas were considered to have high methanogenic potential, and *vice versa*. Based on this dataset, low CH_4/total gas was at least partially negatively related with high forage quality. Plants possessing high forage quality, based on the PCA result, were *C. papaya* (7), *M. esculenta* (17), *M. azedarach* (18), *M. citrifolia* (20) and *S. grandiflora* (26). Plants likely to cause low CH_4/total gas were *A. villosa* (2), *C. hirta* (8), *E. aquea* (11), *M. fragrans* (21) and *S. mahagoni* (27).

4. Discussion

Modifying ruminal fermentation towards lower CH_4 emissions has been receiving attention during the past decade (Beauchemin et al., 2008; McAllister and Newbold, 2008). In this respect, feeding of certain tropical plants, particularly those which contain phenols (*e.g.*, Hess et al., 2008; Soliva et al., 2008) and saponins (*e.g.*, Goel et al., 2008), have been considered to be a promising strategy for mitigating ruminal CH_4 emissions. Despite that, screening of wide range of other available forage plants would be much more efficient when common properties such as different phenolic fractions could be identified and successfully utilized. The present study focused on determining which phenolic fractions contribute to low CH_4 production by using plant material varying largely in these constituents. As a statistical analytical tool for a detailed description of the relationship between the relevant variables (*i.e.*, low methanogenic potential at high forage value) and the positioning of the individual plant in this scheme, the PCA technique was examined for its utility.

4.1. Influence of plant phenolic and fiber fractions on CH_4 production potential

Leaves of *S. mahagoni*, *A. villosa*, *E. aquea*, *M. fragrans*, and *C. hirta* were found promising for reducing the proportion of CH_4 of total gas formation when incubating the plants *in vitro* in a ruminal fluid buffer mixture. All of them contained substantial amounts of phenolic compounds. This supports previous studies which reported that ruminal CH_4 production may be lower when using diets containing phenols (Carulla et al., 2005; Puchala et al., 2005; Animut et al., 2008). But this is not always the case (Beauchemin et al., 2007; Oliveira et al., 2007), and such differences between studies may be related to the diversity in the structures of phenolic compounds, the activities of the individual phenolic sources, interaction with other compounds and the dosages (Makkar, 2003b; Rochfort et al., 2008).

In our study, TP had the closest relationship to CH_4/digestible OM likely because TP includes all phenolic fractions which all were found to contribute to the decrease in CH_4/digestible OM. This finding agrees with Jayanegara et al. (2009b) who found that, compared to TT and CT, TP had a closer relationship with CH_4 reduction, and that TP were more close associated than TT and CT, with the CH_4 increase occurring when the tannin binding agent polyethylene glycol was added. This suggests

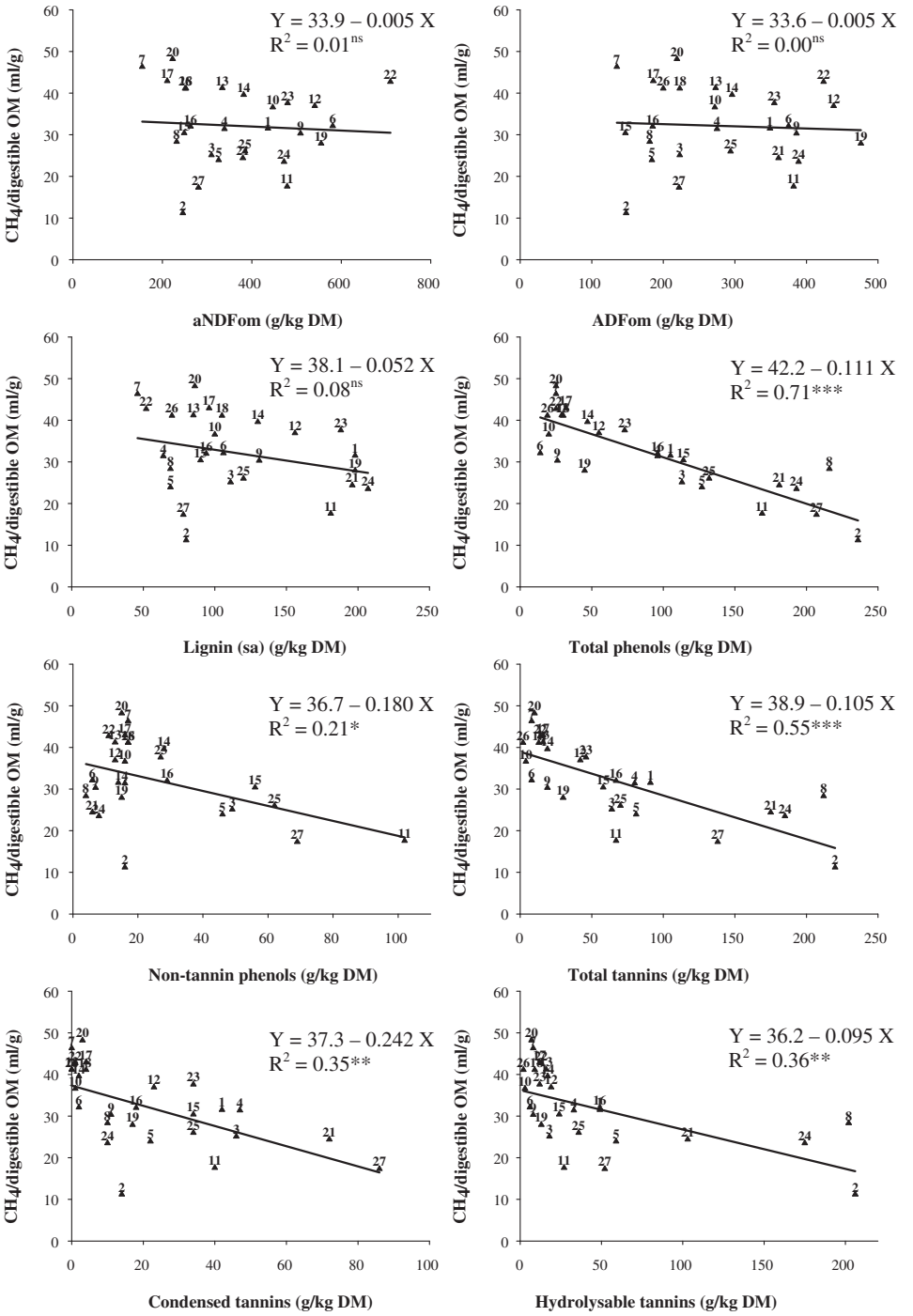

Fig. 1. Regressions of CH_4 production per unit of organic matter digested (in ml/g) on either fiber or phenolic fractions (in g/kg DM). *P<0.05; **P<0.01; ***P<0.001; ns, not significant. The number codes refer to plant species (Table 1).

that TP may be particularly useful for a general screening for plants possessing antimethanogenic activity since, in addition, determination of TP is relatively simple with standard laboratory equipment (Makkar, 2003a). Our results strongly suggest that TT and NTP fractions are both associated with a lower CH_4 emission. However, the mechanisms through which TT and NTP decrease CH_4 appear to differ.

The decrease in CH_4/digestible OM occurring with increasing TT was accompanied by declines in ammonia, total SCFA, total gas and IVOMD. This suggests that at least a part of the CH_4 suppression by TT was due to simultaneously lower degradation of OM, as tannins may interact with dietary protein and fiber thus retarding their digestion (McSweeney et al., 2001;

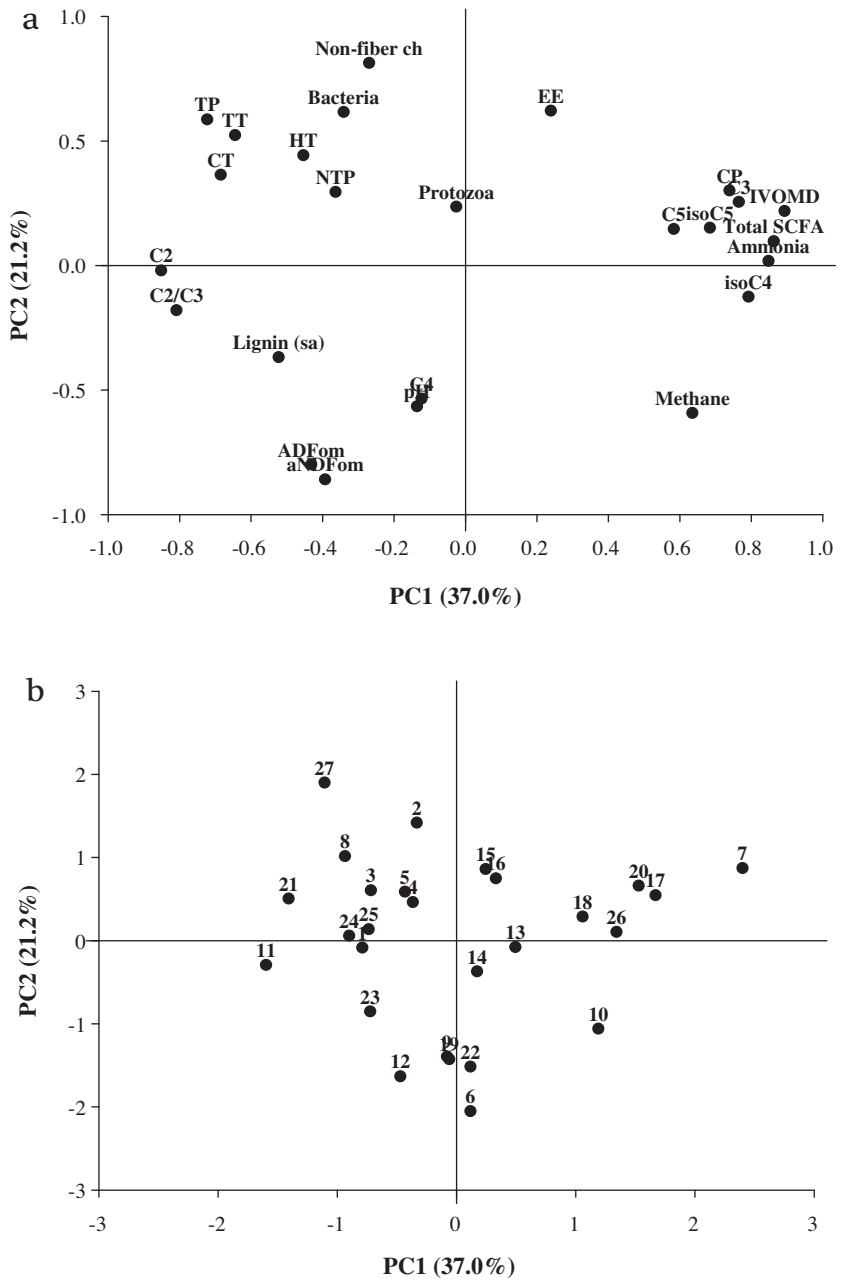

Fig. 2. Plot of the first two PC loading vectors, describing the relationship among variables of plant composition and of ruminal fermentation (a), and plot of the first two PC score vectors, describing the classification of each experimental plant within the PC loading vectors (b). ADFom, acid detergent fiber; C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_2/C_3, acetate/propionate ratio; CP, crude protein; CT, condensed tannins; EE, ether extract; HT, hydrolysable tannins; IVOMD, *in vitro* organic matter digestibility; aNDFom, neutral detergent fiber; Non-fiber ch, non-fiber carbohydrates; NTP, non-tannin phenols; SCFA, short-chain fatty acid; TP, total phenols; TT, total tannins. The number codes refer to plant species (Table 1).

Mueller-Harvey, 2006; Tiemann et al., 2008). Concerning tannin fractions, almost all fermentation parameters declined when CT levels were increased. A similar pattern was observed for HT, except that there was no decrease in ammonia concentration and *iso* SCFA. These variables reflect the extent of ruminal protein degradation (Getachew et al., 2004). It remains unclear if this also reflects differences in the mode of action against ruminal CH_4 production. There are large structural differences between these two tannin fractions. The HT contain carbohydrates (generally D-glucose) as a central core with the hydroxyl groups esterified with phenolic groups. Differing from that, CT do not have this core but are complexes of oligomers and polymers of flavanoid units linked by carbon–carbon bonds and have molecular weights of 2000–4000 kDa (Goel et al., 2005). Ruminal microbes are capable of degrading HT to glucose and gallic acid, but cannot degrade CT (McSweeney et al., 2001; Makkar et al., 2007). Therefore, the apparently lower magnitude in antimethanogenic effect with HT, compared to

CT, may be due to degradation of HT and the associated release of some HT-nutrient complexes. Additionally, the potential toxicity of larger quantities of HT is a serious constraint to its feeding (McSweeney et al., 2001). This toxicity is assumed to be due to absorption of products from HT degradation and the concomitantly higher metabolic load with phenols, which might exceed the detoxification capacity of the liver (Makkar et al., 2007). Methane reduction mediated by a decrease of protozoa (Vogels et al., 1980; Makkar et al., 1995) seems not to have occurred in our study as there were no correlations between either CT or HT and protozoal count. In the study of Śliwiński et al. (2002) an extract containing HT also had no effect on protozoal count. Differing from that, Bhatta et al. (2009) observed a reduction in protozoal count by HT additions.

Differing from tannins, the influence of the NTP on methanogenesis was not accompanied by a generally negative effect on fermentation and protein digestion, which might suggest that the action of NTP is by direct inhibition of the population, or activity of the methanogens, or both (Tavendale et al., 2005). Accordingly, supplementation of hay with 5 mM caffeic, p-coumaric, ferulic and cinnamic acid, which are simple non-tanniniferous phenolic acids, decreased *in vitro* CH_4 production without impairing IVOMD (Jayanegara et al., 2009a). In that study, the order of these phenols in their ability to reduce CH_4 was related to their chemical structure since those with two or more hydroxyl groups seemed to have a higher efficiency than those containing only one. Therefore, it might be worthwhile to isolate the NTP fraction from plants having potential to reduce ruminal CH_4 production, and analyze its composition, in order to obtain a better insight into the role of these phenolic fractions in CH_4 mitigation. There is additional interest in this group of compounds because some of them could exhibit antioxidative properties (Makkar et al., 2007).

Dietary cell wall content and ruminal CH_4 production are considered to be positively correlated since fiber degradation results in large amounts of hydrogen which is utilized by methanogens for CH_4 formation (Machmüller et al., 2003). Consistent with that, CH_4 emissions may decline with increasing dietary proportions of concentrate. However Blaxter and Clapperton (1969), as well as Lovett et al. (2003), found the relationship between a reduced dietary forage to concentrate ratio and CH_4 to be non-linear. It seems that the effect of the prevalence of phenolic compounds is overriding that of fiber which might explain the lack of a significant relationship between aNDFom, ADFom or lignin(sa) and CH_4 per unit digestible OM. Total phenols alone explained 71% of the variation in CH_4/digestible OM.

4.2. Applicability of PCA for assessing relationships between variables and for screening of plants

The PCA technique is a method that is used in the field of animal nutrition and feed science. Examples are the exploration of differences in alkane profiles among plant species (Ferreira et al., 2009), description of relationships among a number of quantitative variables in feeds and their ruminal fermentation pattern (Agbagla-Dohnani et al., 2001), classification of animal fats, fish oils and recycled cooking oils before use in animal nutrition (Van Ruth et al., 2010), and assessment of relationships among individual milk fatty acids under various nutritional regimes (Kadegowda et al., 2008). However, to our knowledge, PCA has not been used in screening plants for low CH_4 production potential at simultaneously acceptable forage quality.

The loading plot of PCA clearly demonstrated a relationship between the plant compositional and the fermentation-related variables measured in our experiment. Commonly, such relationships are presented as a correlation matrix (Ammar et al., 2004; Karabulut et al., 2007) as we did. However, this attempt is restricted to pairwise comparisons, and even multiple regressions are limited in giving a clear ranking across many variables. Using the loading plot of PCA, all variables which are correlated are illustrated in a way which is easy to conceive as they are either arranged in the same direction or in the opposite direction. Moreover, it is possible to assess the magnitude of importance of each variable by the distance from the origin (Everitt, 2005; Härdle and Simar, 2007). Accordingly, in our study, an inverse relationship between CH_4 to total gas production and phenolic fractions evolved from PCA. These phenolic fractions were clustered, showing that they were effective in the same direction. When arranging the fractions according to their distance from the origin, the order of importance was TP > TT > CT = HT > NTP. The comparison with the correlation matrix shows that the order is the same. This again suggests that the loading plot of PCA could replace, at least to a certain extent, the much more difficult to conceive correlation matrix.

The cluster of variables that positively contributes to good tropical forage nutritional quality is in agreement with previous studies. These include positive correlations between CP and NH_3 in the rumen (Getachew et al., 2004) and between CP and IVOMD or total gas production (Larbi et al., 1998; Karabulut et al., 2007). A positive relationship with branched-chain SCFA (*iso* C_4 and *iso* C_5) on forage quality was expected, since these fatty acids are microbial degradation products of branched-chain amino acids such as valine and leucine, and are known to be growth promoting, especially for fiber degrading bacteria (Owens and Bergen, 1983). The inverse directions found in PCA between fiber fractions or phenolic compounds and NH_3, total SCFA, *iso* C_4, total gas or IVOMD is also consistent with other studies (Abdulrazak et al., 2000; Barahona et al., 2003; Gasmi-Boubaker et al., 2005).

Examining the loading plot in more detail, a high CH_4 proportion of total gas seems to have been partially associated with variables related to high quality forage. This implies that there is little probability in finding many plants with both very low CH_4 production potential and high forage quality, but rather a compromise between these two categories has to be found. Indeed, in our study no plant was identified where there was an overlap of low CH_4 and high quality. A possible solution for this problem might be to mix plant possessing a low CH_4 potential and plant possessing a high quality. However, this

association between CH_4 production and forage quality needs to be further investigated using a larger set of plant samples and variables measured. Moreover, such associations need to be tested under *in vivo* conditions as well.

5. Conclusions

All phenolic fractions were negatively correlated with CH_4 emissions in relation to total gas production when feeds were incubated *in vitro* with ruminal fluid. Among these fractions, total phenols had the closest relationship and may be used in screening of a large number of plants for anti-methanogenic properties, partly because its determination is relatively simple. The relationship between low CH_4 and high non-tannin phenols warrants further investigation as this was not accompanied by a general reduction of the intensity of fermentation. Among tannins, a closer look at hydrolysable tannins might be interesting as well, as they have a less adverse effect on protein degradation than condensed tannins, but they may be toxic at higher levels. Principal component analysis proved to be helpful in dealing with a large number of variables and plants, thus facilitating screening of plants for low methanogenic potential and high forage quality. In doing so, from the plants investigated, those possessing good forage quality were *C. papaya*, *M. esculenta*, *M. citrifolia*, *S. grandiflora* and *M. azedarach*, and those having a low CH_4 production potential were *S. mahagoni*, *A. villosa*, *E. aquea*, *M. fragrans* and *C. hirta*. However, the practical utility of these plants, or combinations of them, needs further investigation under long-term *in vivo* conditions.

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References

Abdulrazak, S.A., Fujihara, T., Ondiek, J.K., Ørskov, E.R., 2000. Nutritive evaluation of some Acacia tree leaves from Kenya. Anim. Feed Sci. Technol. 85, 89–98.
Agbagla-Dohnani, A., Noziere, P., Clement, G., Doreau, M., 2001. *In sacco* degradability, chemical and morphological composition of 15 varieties of European rice straw. Anim. Feed Sci. Technol. 94, 15–27.
Ammar, H., Lopez, S., Gonzalez, J.S., Ranilla, M.J., 2004. Chemical composition and *in vitro* digestibility of some Spanish browse plant species. J. Sci. Food Agric. 84, 197–204.
Animut, G., Puchala, R., Goetsch, A.L., Patra, A.K., Sahlu, T., Varel, V.H., Wells, J., 2008. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Anim. Feed Sci. Technol. 144, 212–227.
AOAC, 1997. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, VA, USA.
Barahona, R., Lascano, C.E., Narvaez, N., Owen, E., Morris, P., Theodorou, M.K., 2003. *In vitro* degradability of mature and immature leaves of tropical forage legumes differing in condensed tannin and non-starch polysaccharide content and composition. J. Sci. Food Agric. 83, 1256–1266.
Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85, 1990–1996.
Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAlister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21–27.
Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512–5522.
Blaxter, K.L., Clapperton, L., 1969. Prediction of the amount of methane produced by ruminants. Brit. J. Nutr. 19, 511–522.
Bodas, R., Lopez, S., Fernandez, M., Garcia-Gonzalez, R., Rodriguez, A.B., Wallace, R.J., Gonzalez, J.S., 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Anim. Feed Sci. Technol. 145, 245–258.
Carulla, J.E., Kreuzer, M., Machmüller, A., Hess, H.D., 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agric. Res. 56, 961–970.
Ehrlich, G.G., Goerlitz, D.F., Bourell, J.H., Eisen, G.V., Godsy, E.M., 1981. Liquid chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. Appl. Environ. Microbiol. 42, 878–886.
EPA (United States Environmental Protection Agency), 2010. Methane and Nitrous Oxide Emissions from Natural Sources. United States Environmental Protection Agency, Office of Atmospheric Programs, Washington, DC, USA, http://www.epa.gov/methane/pdfs/Methane-and-Nitrous-Oxide-Emissions-From-Natural-Sources.pdf.
Evans, J.D., Martin, S.A., 2000. Effects of thymol on ruminal microorganisms. Curr. Microbiol. 41, 336–340.
Everitt, B.S., 2005. An R and S-Plus Companion to Multivariate Analysis. Springer-Verlag, London, UK.
Ferreira, L.M.M., Celaya, R., Garcia, U., Rodrigues, M.A.M., Osoro, K., 2009. Differences between domestic herbivores species in alkane faecal recoveries and the accuracy of subsequent estimates of diet composition. Anim. Feed Sci. Technol. 151, 128–142.
Garcia-Gonzalez, R., Lopez, S., Fernandez, M., Bodas, R., Gonzalez, J.S., 2008. Screening the activity of plants and spices for decreasing ruminal methane production *in vitro*. Anim. Feed Sci. Technol. 147, 36–52.
Gasmi-Boubaker, A., Kayouli, C., Buldgen, A., 2005. *In vitro* gas production and its relationship to *in situ* disappearance and chemical composition of some Mediterranean browse species. Anim. Feed Sci. Technol. 123–124, 303–311.
Getachew, G., Robinson, P.H., DePeters, E.J., Taylor, S.J., 2004. Relationships between chemical composition, dry matter degradation and *in vitro* gas production of several ruminant feeds. Anim. Feed Sci. Technol. 111, 57–71.
Goel, G., Puniya, A.K., Aguilar, C.N., Singh, K., 2005. Interaction of gut microflora with tannins in feeds. Naturwissenschaften 92, 497–503.
Goel, G., Makkar, H.P.S., Becker, K., 2008. Effects of *Sesbania sesban* and *Carduus pycnocephalus* leaves and Fenugreek (*Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Anim. Feed Sci. Technol. 147, 72–89.
Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C., Eckard, R.J., 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89, 241–251.
Härdle, W., Simar, L., 2007. Applied Multivariate Statistical Analysis, Second Edition. Springer-Verlag, Berlin, Germany.
Hess, H.D., Mera, M.L., Tiemann, T.T., Lascano, C.E., Kreuzer, M., 2008. *In vitro* assessment of the suitability of replacing the low-tannin legume *Vigna unguiculata* with the tanniniferous legume *Leucaena leucocephala*, *Flemingia macrophylla* or *Calliandra calothyrsus* in a tropical grass diet. Anim. Feed Sci. Technol. 147, 105–115.
IPCC (Intergovernmental Panel on Climate Change), 2006. Guidelines for National Greenhouse Gas Inventories. Vol. 4. Agriculture, Forestry and Other Land Use. Ch. 10. Emissions from Livestock and Manure Management, pp. 10.1–10.87.

Jayanegara, A., Makkar, H.P.S., Becker, K., 2009a. Methane reduction effect of simple phenolic acids evaluated by *in vitro* Hohenheim gas production method. Proc. Soc. Nutr. Physiol. 18, 98.
Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009b. Tannins determined by various methods as predictors of methane production reduction potential of plants by an *in vitro* rumen fermentation system. Anim. Feed Sci. Technol. 150, 230–237.
Kadegowda, A.K.G., Piperova, L.S., Erdman, R.A., 2008. Principal component and multivariate analysis of milk long-chain fatty acid composition during diet-induced milk fat depression. J. Dairy Sci. 91, 749–759.
Kaiser, H.F., 1960. The application of electronic computers to factor analysis. Educ. Psychol. Meas. 20, 141–151.
Karabulut, A., Canbolat, O., Kalkan, H., Gurbuzol, F., Sucu, E., Filya, I., 2007. Comparison of *in vitro* gas production, metabolizable energy, organic matter digestibility and microbial protein production of some legume hays. Asian-Aust. J. Anim. Sci. 20, 517–522.
Larbi, A., Smith, J.W., Kurdi, I.O., Adekunle, I.O., Raji, A.M., Ladipo, D.O., 1998. Chemical composition, rumen degradation, and gas production characteristics of some multipurpose fodder trees and shrubs during wet and dry seasons in the humid tropics. Anim. Feed Sci. Technol. 72, 81–96.
Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P., 2003. Effect of forage/concentrate ration and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest. Prod. Sci. 84, 135–146.
Machmüller, A., Soliva, C.R., Kreuzer, M., 2003. Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. Brit. J. Nutr. 90, 529–540.
Makkar, H.P.S., Blümmel, M., Becker, K., 1995. *In vitro* effects of and interactions between tannins and saponins and the fate of tannins in the rumen. J. Sci. Food Agric. 69, 481–493.
Makkar, H.P.S., 2003a. Quantification of Tannins in Tree and Shrub Foliage: A Laboratory Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Makkar, H.P.S., 2003b. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rum. Res. 49, 241–256.
Makkar, H.P.S., Francis, G., Becker, K., 2007. Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. Animal 1, 1371–1391.
Mascarelli, A.L., 2009. A sleeping giant? Nat. Rep. 3, 46–49, www.nature.com/reports/climatechange.
McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7–13.
McCrabb, G.J., Hunter, R.A., 1999. Prediction of methane emissions from beef cattle in tropical production systems. Aust. J. Agric. Res. 50, 1335–1340.
Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of the digestibility and metabolizable energy content of ruminant feeding-stuff from the gas production when they are incubated with rumen liquor *in vitro*. J. Agric. Sci. 93, 217–222.
McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O., 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91, 83–93.
Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.
Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010–2037.
Oliveira, S.G., Berchielli, T.T., Pedreira, M.S., Primavesi, O., Frighetto, R., Lima, M.A., 2007. Effect of tannin levels in sorghum silage and concentrate supplementation on apparent digestibility and methane emission in beef cattle. Anim. Feed Sci. Technol. 135, 236–248.
Owens, F.N., Bergen, W.G., 1983. Nitrogen metabolism of ruminant animals: historical perspective, current understanding and future implications. J. Anim. Sci. 57, 498–518.
Puchala, R., Min, B.R., Goetsch, A.L., Sahlu, T., 2005. The effect of a condensed tannin-containing forage on methane emission by goats. J. Anim. Sci. 83, 182–186.
Rochfort, S., Parker, A.J., Dunshea, F.R., 2008. Plant bioactives for ruminant health and productivity. Phytochemistry 69, 299–322.
SAS Institute Inc., 2008. SAS/STAT Software, Version 9.2. SAS Institute Inc., Cary, NC, USA.
SigmaPlot, 2008. SigmaPlot Software, Version 11.0. Systat Software, Inc., San Jose, CA, USA.
Singh, B., Sahoo, A., Sharma, R., Bhat, T.K., 2005. Effect of polyethylene glycol on gas production parameters and nitrogen disappearance of some tree forages. Anim. Feed Sci. Technol. 123–124, 351–364.
Śliwiński, B.J., Kreuzer, M., Wettstein, H-.R., Machmüller, A., 2002. Rumen fermentation and nitrogen balance of lambs fed diets containing plant extracts rich in tannins and saponins, and associated emissions of nitrogen and methane. Arch. Anim. Nutr. 56, 379–392.
Soliva, C.R., Hess, H.D., 2007. Measuring methane emission of ruminants by *in vitro* and *in vivo* techniques. In: Makkar, H.P.S., Vercoe, P.E. (Eds.), Measuring Methane Production from Ruminants. Springer, Dordrecht, The Netherlands, pp. 15–31.
Soliva, C.R., Zeleke, A.B., Clement, C., Hess, H.D., Fievez, V., Kreuzer, M., 2008. *In vitro* screening of various tropical foliages, seeds, fruits and medicinal plants for low methane and high ammonia generating potentials in the rumen. Anim. Feed Sci. Technol. 147, 53–71.
Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Technol. 123–124, 403–419.
Tiemann, T.T., Lascano, C.E., Kreuzer, M., Hess, H.D., 2008. The ruminal degradability of fibre explains part of the low nutritional value and reduced methanogenesis in highly tanniniferous tropical legumes. J. Sci. Food Agric. 88, 1794–1803.
Van Ruth, S.M., Rozijn, M., Koot, A., Perez-Garcia, R., Van der Kamp, H., Codony, R., 2010. Authentication of feeding fats: classification of animal fats, fish oils and recycled cooking oils. Anim. Feed Sci. Technol. 155, 65–73.
Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.
Vogels, G.D., Hoppe, W.F., Stumm, C.K., 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40, 608–612.
Vogt, T., 2010. Phenylpropanoid biosynthesis. Mol. Plant 3, 2–20.