In vitro methane and gas production characteristics of *Eragrostis trichopophora* substrate supplemented with different browse foliage

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Abstract

An *in vitro* gas production study was conducted to evaluate the potential of six browse species (high, medium and low condensed tannin concentrations) collected from the Kalahari Desert as antimethanogenic additives to an *Eragrostis trichopophora*-based substrate. The browse species studied were *Acacia luederitzii*, *Monechma incanum*, *Acacia erioloba*, *Acacia haematoxylon*, *Olea europaea* and *Acacia mellifera*. The edible forage dry matter of the browse species were incubated with *Eragrostis trichopophora* in a 30 : 70 (w/w) ratio by adding 40 mL of a buffered rumen fluid at 39°C for 48 h. Gas and methane production at different time intervals after incubation were determined whereas the volatile fatty acids concentration was evaluated after 48 h. *Acacia luederitzii* and *M. incanum* foliage decreased methane production by more than 50%, but simultaneously decreased digestibility, and rumen fermentation parameters such as volatile fatty acids concentration. Tannin extracts from *A. luederitzii* could possibly be used as a dietary alternative to reduce methane production; however, there is a need to determine an optimum level of inclusion that may not compromise the efficiency of rumen fermentation and overall digestibility of the diet.

Additional keywords: digestibility, tannin.

Introduction

Ruminants are major contributors to biogenic methane (CH_4) production, and it has been estimated that the reduction of CH_4 formation from domesticated ruminants could contribute to stabilising atmospheric CH_4 concentrations (Gibbs *et al*. 1989; Crutzen 1995; Johnson and Johnson 1995). Reducing enteric CH_4 has become a focus of animal nutrition, especially in countries where agriculture is a major economic enterprise. There are currently no robust, reproducible and economically viable methods for reducing CH_4 emissions from ruminants grazing on pastures. Manipulating the rumen microbial ecosystem to enhance digestibility of fibrous feeds, reduce CH_4 emission and nitrogen (N) excretion, and also improve performance, are some of the most important goals of animal nutritionists.

However, researchers manipulating the rumen microbial ecosystem to enhance digestibility of fibrous feeds, and reduce CH_4 emission and N excretion by ruminants have failed to find an effective chemical inhibitor of ruminal CH_4 formation whose efficacy will persist for several days (Clapperton 1977; Van Nevel and Demeyer 1996). The only effective chemical inhibitor widely in use is ionophores, which inhibits the formation of free hydrogen by species that provides hydrogen to the methanogens (Nagaraja *et al*. 1997), and subsequently decrease CH_4 emissions by up to 25% (Van Nevel and Demeyer 1996), but the overall effect of ionophores appears to be inconsistent (Nagaraja *et al*. 1997). There is a need for feed additives with the potential to reduce ruminal methanogenesis. Extensive screening of plants and plant extracts that exhibit CH_4-reducing properties have been conducted (Kamra *et al*. 2006; Bodas *et al*. 2008; García-González *et al*. 2008; Soliva *et al*. 2008). Tannins are among the compounds considered promising in CH_4 reduction (Patra and Saxena 2010). In tropical herbaceous forages like trees and shrub species, there are appreciable amounts of tannins and other phenolic compounds in their foliage, which may reduce CH_4 production (Martin *et al*. 2010).

However, the effectiveness of plants and plant extracts that have high levels of saponins, flavonoids and tannins, varies depending on the molecular weight, type and concentration of these compounds (Patra *et al*. 2006). Some *in vitro* studies combined additives with single substrates such as grain meals (Callaway and Martin 1996; Carro and Ranilla 2003; Pellikaan *et al*. 2011) or hay (Lourenco *et al*. 2008; Goel *et al*. 2009) to reduce CH_4 production whereas other studies used a mixed basal substrate, such as alfalfa hay (Wang *et al*. 2000; Busquet *et al*. 2005) or grass hay (Lila *et al*. 2003; Hu *et al*. 2005; Guo *et al*. 2008) combined with a concentrate in evaluating CH_4 reduction. Few reports exist in which the effects of additives were studied in combination with different substrates within a single experiment to reduce enteric CH_4 emissions. Research to identify new compounds or novel uses for existing natural products to reduce CH_4 is expensive, but is essential to identify new active compounds given the wide range of molecular diversity in these products (Borris 1996). The aim of this study was to evaluate the effect of supplementing *E. trichopophora* grass with browse species containing high, medium and low levels of tannins at 70 : 30 ratio with or without polyethylene glycol (PEG), on CH_4 production and digestibility. In doing so, we can possibly select browse species with the potential to decrease CH_4 production in extensive farming systems.

Materials and methods

Selection of the browse species

One kilogram of edible foliage sample of six browse species were sampled in the Kalahari (26°46.610'S, 22°34.557'E) area situated in the Northern Cape Province of South Africa between March and April 2012. These species (*M. incanum*, *A. luederitzii*, *A. erioloba*, *A. haematoxylon*, *O. europaea* and *A. mellifera*) were analysed for total tannin and condensed tannin (CT) concentrations and based on its tannin concentrations (data not included) were selected to be used in this study. Two browse species each representing a low, medium and high tannin concentration group, were selected for inclusion and incubated with the substrate *E. trichopophora* in a 30 : 70 ratio (w/w).

In vitro gas production measurement

Collection of rumen fluid from donor sheep. Rumen fluid was collected before the morning feeding from two rumen-cannulated Merino wethers fed alfalfa hay *ad libitum*. Approximately 500 mL of the rumen fluid was collected from each donor animal, mixed, strained through four layers of cheesecloth and transferred inside a pre-warmed thermos flask to the laboratory (Grant and Mertens 1992). After blending, the rumen fluid was transferred to a large glass beaker inside a 39°C water bath being continuously purged with carbon dioxide and under continuous stirring as recommended by Goering and Van Soest (1970). Thereafter, the required volume of rumen fluid was added to the buffer solution in the respective incubation bottles; 15 mL of rumen fluid to 25-mL parts of buffer solution.

Buffer media preparation, *sample incubation and gas measurement*. The buffer solution, macro and micro mineral solution was prepared as described in Goering and Van Soest (1970) with the modifications suggested by Mould *et al*. (2005). A semi-automated system was used to measure gas production through *in vitro* incubation at 39°C, according to the method described by Theodorou *et al*. (1994). The system consists of a digital data tracker (Tracker 220 series indicators, Omega Engineering, Inc., Laval, QC, Canada), connected to a pressure transducer (PX4200–015GI from Omega Engineering, Inc.) with a needle tip. Approximately 400 mg of respective substrate was weighed into a 120-mL serum bottle. Thereafter, 40 mL of rumen fluid and buffer media was added under a stream of carbon dioxide to each serum bottle and then closed with a rubber stopper and a crimp seal cap. A needle was inserted through the rubber stopper of each serum bottle for ~5 s to release the small amount of gas that might have built up and create the starting point for incubation. All serum bottles were returned to the incubator (39°C) and the rotary shaker was turned on at 120 rpm. Gas pressure was taken at 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 72 h after incubation. To quantify gas production derived from the culture medium and the ruminal inoculums, two blanks were included in every analysis. Two replicates of the same browse and four different cycles were executed for every browse sample studied. The pressure and volume values were registered and added to the values of the previous readings. Therefore, the cumulative pressure and volume of the fermentation gases could be obtained. However, fermentation was terminated after 72 h by removing the serum bottles from the incubator and placing them on ice. After opening the bottle, pH readings were taken and the supernatants were collected and stored for volatile fatty acids (VFA) analyses.

Short-chain VFA analyses

From each incubation bottle, 5 mL of supernatant was collected and stored for analysis of VFA. Samples were centrifuged in a Sorvall centrifuge (SL-50 T, 8 × 50 mL) at 25 000*g* for 15 min at 4°C and a part of the supernatant was transferred to a micro-centrifuge tube (capacity 1.5 mL) containing meta-phosphoric acid (250 g/L). The standard VFA mixture consisted of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids was used as internal standard, and was treated in the same manner as that for the sample. The VFA in the test sample was analysed using a gas chromatograph with flame ionisation detector (FID) analyser, calibrated against the standard. The final concentration was reported after deducting the corresponding blank values.

Determination of *in vitro* organic matter digestibility (IVOMD)

The procedure used in this study was according to that of Tilley and Terry (1963), as modified by Engels and Van der Merwe (1967), which involves two digestion phases. During the first digestion phase, feed samples (200 mg) were incubated in triplicate under anaerobic conditions with rumen liquor for 48 h at 39°C with the inclusion of blanks and standards in every batch of incubation. This was followed by a 48-h acid pepsin digestion phase at 39°C under anaerobic conditions. Following the 96-h incubation, the residual plant materials were collected and oven-dried at 105°C for 12 h. Ash contents were determined by combustion (550°C for 2 h) (Engels and Van der Merwe 1967). Metabolisable energy was determined by using the equation ME = 0.016*IVOMD suggested by McDonald *et al*. (2002) for roughages.

Methane production measurements

Methane production was measured from duplicate bottles incubated for each substrate by taking gas samples at 2 h, 12 h, 24 h, and 48 h after incubation and analysing the CH_4 concentration using a gas chromatograph (8610C BTU gas analyser gas chromatograph system; SRI Instruments GmbH, Bad Honnef, Germany) calibrated using standard analytical gas. Gas produced from each bottle at various times was recorded and samples of the gas were taken using a Hamilton syringe and 1 mL of sampled gas produced was injected manually into the gas chromatograph. Two blanks were included for correction of CH_4 produced from the inoculum in each cycle and a total of two cycles were executed. The CH_4 concentration that was measured at each time interval was related to total gas volume to determine its concentration (Tavendale *et al*. 2005) and converted later into energy and mass values using 39.54 kJ/L CH_4 and 0.716 mg/mL CH_4 factors, respectively (Santoso *et al*. 2007).

Statistical analyses

Grass hay nutrient composition, gas and CH_4 production and VFA concentrations were statistically analysed using the GLM option of SAS (2010), and mean differences among foliage species were determined using Duncan's multiple-range test.

Results and discussion

Chemical composition

The chemical composition of *E. trichopophora* used as test feed have the following composition: ash (33.1 g/kg DM), crude protein (34.3 g/kg DM), ether extract (9.5 g/kg DM), neutral detergent fibre (794.9 g/kg DM), acid detergent fibre (477.7 g/kg DM), acid detergent fibre (67.9 g/kg DM) and neutral detergent-insoluble N (21 g/kg DM). *Eragrostis trichopophora* is low in crude protein with a high neutral detergent fibre concentration.

Table 1. Volumes (mL/g DM) of gas production from the studied browse and shrubs supplemented to grass hay at a ratio of 30 : 70 with or without polyethylene glycol (PEG)

Means with different lower-case letters within a column are significantly ($P < 0.001$) different. Means with different upper-case letters in rows within each incubation time are significantly ($P < 0.05$) different. No PEG, denotes presence of tannin; PEG, denotes absence of tannin

		Gas production (mL/g DM)							
		2 h		12 h		24 h		48 h	
Species	Tannin level	PEG	No PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG
Acacia luederitzii	High	22.1aA	5.2dB	77.1abA	39.8dB	166.2aA	115.5eA	227.9aA	147.7cB
Monechma incanum	High	18.2bA	9.5cB	79.4aA	50.1bB	163.9aA	134.2cB	212.1bA	161.7dB
Acacia erioloba	Medium	21.3aA	17.5aB	73.5bA	54.5abB	141.0bA	131.4dB	188.6dA	160.3dB
Acacia haematoxylon	Medium	19.6abA	13.0bB	78.1abA	56.3aB	161.2aA	132.3dB	197.1cA	177.2bB
Olea europaea	Low	11.8dB	17.0aA	58.8cA	50.3bB	125.6cA	145.5bB	164.4fA	157.8cbB
Acacia mellifera	Low	14.7cA	12.0cbB	55.0cA	44.5cB	120.9cA	143.6bB	179.8eA	163.8cbB
Eragrostis trichopophora	No tannin	4.4eA	3.5dB	57.2cA	45.3cB	157.5aA	156.0aA	221.8aA	212.9aB
s.e.m.		0.39	0.34	0.66	0.62	1.85	1.00	1.01	1.33
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*In vitro g*as production and IVOMD

There were significant differences ($P < 0.001$) among the different browses included in the grass-browse substrate in terms of cumulative gas production when incubated with or without PEG as a tannin binder (Table 1). The effects of tannin ('PEG' versus 'No PEG') in the grass-browse substrates, showed that gas production was reduced by 40 mL and 65 mL in substrate with high CT browse, 19 mL and 24 mL in substrate with medium CT browse and 9 mL and 11 mL in low CT browse after 24 h and 48 h incubation, respectively. *Acacia luederitzii* and *M. incanum* (high tannin) decreased gas production by an average of 20% whereas *A. erioloba* and *A. haematoxylon* (medium tannin) decreased gas production by an average of 15.5% whereas *O. europaea* and *A. mellifera* (low tannin) reduced gas production by 7.3% after 24 h of *in vitro* incubation.

Volatile fatty acid concentration

Incubation of the grass with high (*A. luederitzi* and *M. incanum*) and medium (*A. erioloba* and *A. haematoxylon*) CT-containing browse substrate resulted in decreased total VFA production whereas incubation of the grass substrate with low CT-containing browses (*A. melifera* and *O. europa*) led to increased total VFA production. However, the inclusion of PEG as a tannin binder resulted in an increase in VFA concentration for each browse-grass substrate (Table 2). The acetic, propionic, iso butyric, butyric, and valeric acid concentrations differed among the browse species. Supplementation of browse with high and medium tannin concentration to Eragrostis grass hay decreased acetatic acid production, which is a major product of fibre fermentation but no difference was noted in grass supplemented with browse species having low tannin concentrations. The effects of CT on ruminal VFA concentration and composition vary among studies, depending on dose and source of CT (Bhatta *et al*. 2005). In the study of Khiaosa-Ard *et al*. (2009) using a rumen simulation technique, a decrease in the acetate : propionate ratio was reported when CT extract from *A. mearnsii* was fed to animals. Condensed tannins resulted in a decrease in acetate production when compared with the PEG-included incubations in the grass-browse substrates that contain high and medium CT concentrations whereas supplementation of browse species with low CT concentrations resulted in increased acetate production without PEG inclusion. Beauchemin *et al*. (2007) reported that increasing levels of tannins up to 20 g/kg DM tended to decrease the ruminal total VFA concentration and the acetate : propionate ratio in cattle.

In this study, the acetate : propionate ratio decreased when no PEG was included (data not reported in Table 3). In contrast, Carulla *et al*. (2005), Beauchemin *et al*. (2007) and Khiaosa-Ard *et al*. (2009) reported that VFA concentrations remained unchanged, but that the molar proportion of propionate increased in sheep fed *A. mearnsii* containing CT. In general, molar ratios of the principal VFA are not changed by feeding CT-containing forages (Waghorn and Shelton 1997; Puchala *et al*. 2005), but their concentrations in rumen liquor are often reduced, probably a reflection of a larger rumen pool size and a slower rate of VFA production from a slower rate of fibre digestion (Waghorn *et al*. 1994). The results from this study indicate that the concentration of total tannins and CT is negatively related to VFA production and particularly acetate, thus compromising the availability of nutrient for microbial synthesis.

Table 2. Total and individual volatile fatty acid (mmol/L) concentration in the supernatant after 72-h incubation of 400 mg DM of browses with or without polyethylene glycol (PEG)

Means with different lower-case letters within a column are significantly ($P < 0.05$) different. Means with different upper-case letters in rows within each incubation time are significantly ($P < 0.05$) different. No PEG, denotes presence of tannin; PEG, denotes absence of tannin. IVOMD, *in vitro* organic matter digestibility; VFA, volatile fatty acid

	Acetic		Propionic		Iso butyric		Butyric		Valeric		Total VFA mmol/L		IVOMD	
Species	PEG	NO PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG
Acacia luederitzii	51.91eA	49.19dB	12.63eB	13.91cA	1.44eA	1.41dA	5.06fA	4.74fB	1.71eA	1.78cA	72.76fA	71.04gB	58.79aA	47.51dB
Monechma incanum	59.04aA	48.9dB	15.72aA	13.44dB	1.70bA	1.47cB	7.15aA	5.60eB	2.15aA	1.78cB	85.76aA	71.78fB	58.88aA	52.27bB
Acacia erioloba	56.21cA	55.84bA	15.16bA	14.92bA	1.76aA	1.77aA	6.41dA	6.26cA	2.18aA	2.17aA	81.72cA	81.07cB	50.31dA	48.19cB
Acacia haematoxylon	57.68bA	51.50cB	14.85cA	13.23eB	1.74aA	1.64bB	6.58bA	5.88dB	2.11bA	1.96bB	82.96bA	74.21eB	55.73bA	49.31bcB
Olea europaea	50.61fB	55.74bA	14.93cB	15.82aA	1.50dB	1.62bA	6.15eB	6.50bA	1.88dA	1.93bA	74.53eB	81.61bA	45.63fA	43.38fB
Acacia mellifera	54.25dB	61.72aA	14.00dB	15.60abA	1.57cB	1.79aA	6.51cB	7.29aA	1.96cB	2.16aA	78.29dB	88.55aA	47.01eA	45.40eB
Eragrostis trichopophora	62.13aA	56.98bB	15.26abA	13.54cdB	1.63cA	1.42dB	6.20eA	5.82dB	1.83dA	1.76cA	87.05aA	79.52dB	54.74cA	54.4aA

Table 3. Volumes (mL/g DM) of methane produced by browse species with different tannin concentrations supplemented to grass hay at a ratio of 30 : 70 with or without polyethylene glycol

Means with different lower-case letters within a column are significantly ($P < 0.001$) different. Means with different upper-case letters in rows within each incubation time are significantly ($P < 0.05$) different. No PEG, denotes presence of tannin; PEG, denotes absence of tannin

		Methane production (mL/g DM)							
		2 h		12 h		24 h		48 h	
Species	Tannin level	PEG	No PEG	PEG	NO PEG	PEG	No PEG	PEG	NO PEG
Acacia luederitzii	High	0.38aA	0.03dB	4.05abA	0.48dB	14.20cA	6.15dB	23.00cA	8.93dB
Monechma incanum	High	0.35aA	0.10bcB	4.60bA	1.73abcB	17.20abA	14.20bB	25.08cA	16.20bB
Acacia erioloba	Medium	0.38aA	0.25aB	3.83bcA	2.13abB	13.33cdA	10.88cB	18.85dA	14.20cB
Acacia haematoxylon	Medium	0.33abA	0.13bB	3.95abA	1.65bcB	12.28deA	10.53cB	18.55dA	15.88bB
Olea europaea	Low	0.33abA	0.15bB	3.28cdA	2.10abB	11.05efA	10.98cA	18.90dA	16.05bB
Acacia mellifera	Low	0.23bA	0.10bcB	2.68deA	1.18cB	9.33fA	9.08cA	15.78cA	14.80cbB
Eragrostis trichopophora	No tannin	0.10cA	0.05cA	2.80deA	2.28aB	18.78aA	17.63aB	39.93aA	33.95aB
s.e.m.		0.17	0.01	0.089	0.08	0.258	0.309	0.32	0.338
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 4. Ratio of methane to some fermentation parameters produced by browse species with different tannin concentrations supplemented to grass hay at a ratio of 30 : 70 with or without polyethylene glycol

Means with different lower-case letters within a column are significantly ($P < 0.05$) different. Means with different upper-case letters in rows within each incubation time are significantly ($P < 0.05$) different. No PEG, denotes presence of tannin; PEG, denotes absence of tannin. CH_4, methane; IVOMD, *in vitro* organic matter digestibility; VFA, volatile fatty acid

		CH_4/total gas		CH_4/VFA		CH_4/IVOMD	
Species	Tannin level	PEG	No PEG	PEG	No PEG	PEG	No PEG
Acacia luederitzii	High	0.085424bA	0.05327dB	0.195162bA	0.086571cB	0.241538cA	0.129446dB
Monechma incanum	High	0.104955bB	0.105852bB	0.20056abA	0.197827bB	0.29212bA	0.271666bB
Acacia erioloba	Medium	0.094573bA	0.082782bB	0.163118cA	0.134205cB	0.264957cA	0.225773cB
Acacia haematoxylon	Medium	0.076188cB	0.079604cA	0.148023dA	0.141895bB	0.220348dA	0.213547cB
Olea europaea	Low	0.087978bcB	0.095106bA	0.148262dA	0.134542bB	0.242165cB	0.253112bA
Acacia mellifera	Low	0.077184cB	0.07993cA	0.119172eA	0.102541cB	0.198468eB	0.20bA
Eragrostis trichopophora	No tannin	0.119238aA	0.113013aA	0.215738aB	0.221705aA	0.343076aA	0.324081aA

In vitro enteric CH4 production

There were significant differences ($P < 0.001$) between the different browse species in terms of the *in vitro* CH_4 production after 48 h of incubation (Table 3). The result shows that the tannin concentration of the browse species played a significant role in suppressing methanogenic activities. Substrates containing high tannin browse reported greatest reduction in CH_4 production followed by substrate with medium tannin and least in the substrate with low tannin concentration. The relationship between tannin concentration in the browse species and CH_4 production at 48 h *in vitro* was very significant (Fig. 1). The proportion of CH_4 in the total gas produced and the ratio of CH_4 produced to IVOMD was lower with increase in the level of tannin in the browse species as indicated by the differences in CH_4 to total gas ratio between incubations with PEG and those without PEG (Table 4). However, CH_4 produced per unit of total VFA increased with the concentration of tannin in the browse species (Fig. 2).

Fig. 1. Effects of tannin concentration of browse species on 48-h methane production in a browse-grass substrate (30 : 70) with or without polyethylene glycol.

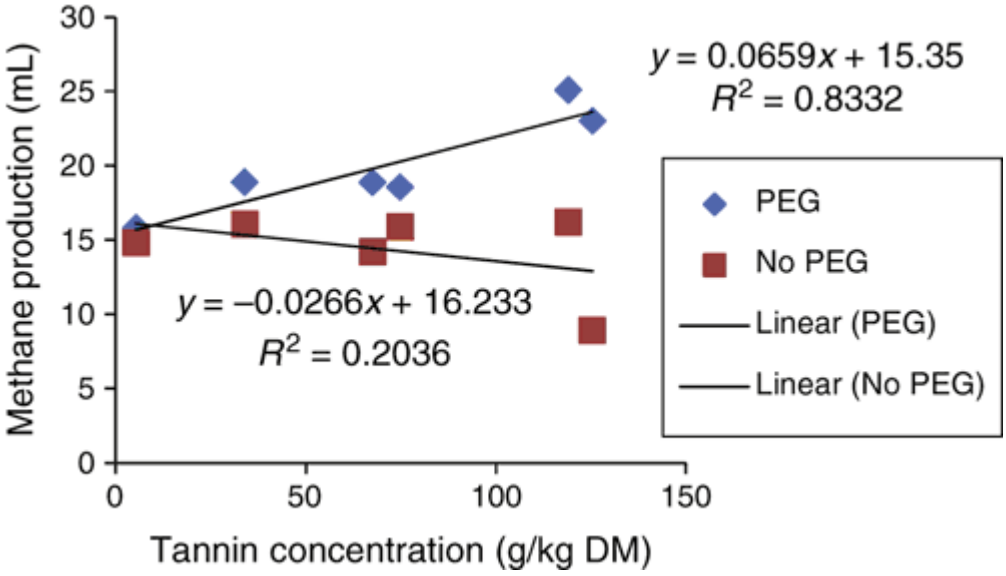
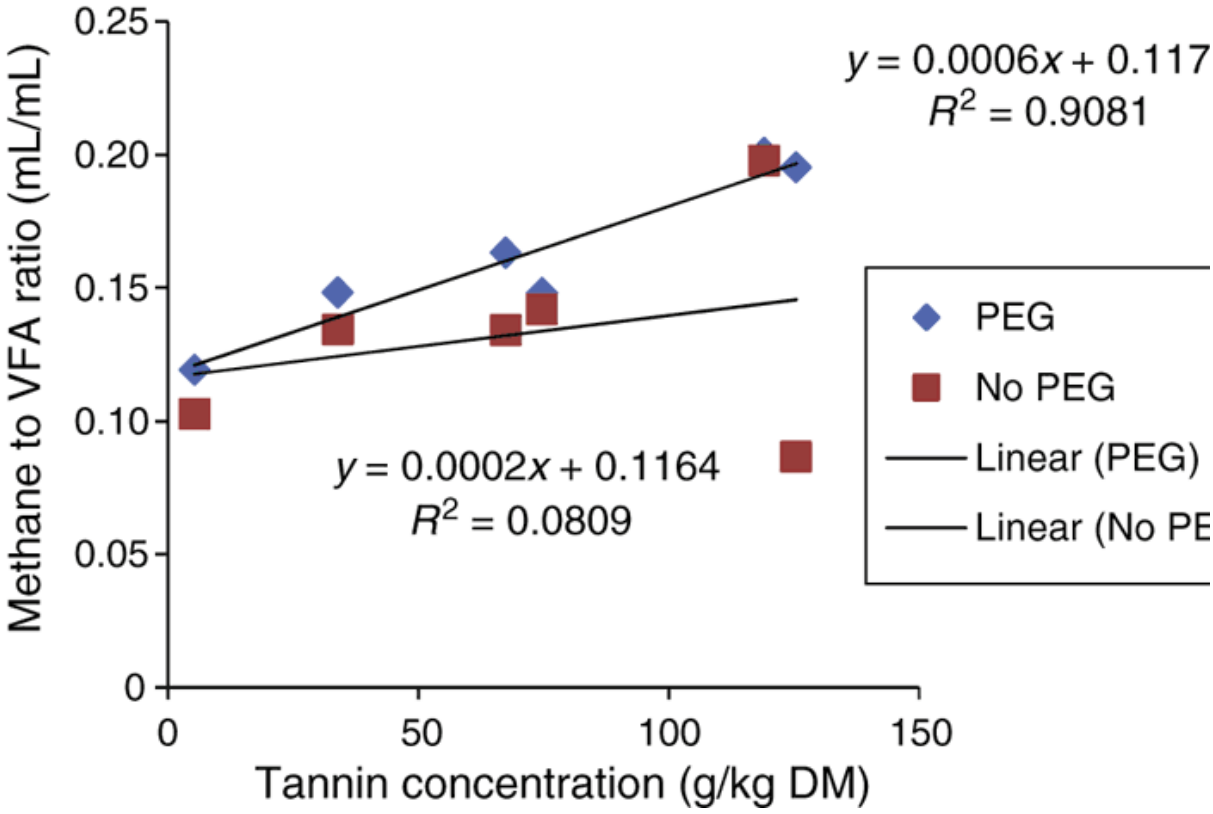


Fig. 2. Effects of tannin concentration of browse species on the ratio of methane to volatile fatty acid production in a browse-grass substrate (30 : 70) with or without polyethylene glycol.

glycol.



The total amount of CH_4 produced after 24 h *in vitro* incubation was decreased by 61.17% in substrate containing *A. luederitzii* and 35.41% in *M. incanum* associated with the inhibitory effects of their tannins on CH_4 whereas *O. europaea* and *A. mellifera* did not show significant effect on CH_4 reduction at 24-h incubation. The cumulative CH_4 production at 48-h incubation was decreased by 61.2%, 43.6%, 24.7% and 14.4% in *A. luederitzii*, *M. incanum*, *A. erioloba* and *A. haematoxylon*, respectively. The efficacy of CH_4 reduction can be associated to their tannin concentrations, which may indicate direct inhibition of methanogenesis, fermentation of organic matter or their inhibitory effects on ciliate protozoa (Hess *et al*. 2003).

The action of CT on methanogenesis has been attributed partly to indirect effects of reduced hydrogen production and organic matter digestibility, and partly by direct inhibitory effects on methanogens (Monforte-Briceño *et al*. 2005; Tavendale *et al*. 2005). Hess *et al*. (2004) reported that *in vitro* CH_4 production decreased when *Calliandra* tannins were supplemented to a tropical grass substrate as was observed when *A. luederitzii* and *M. incanum* was supplemented to *E. trichopophora*. This further confirms earlier reports that CT will lower CH_4 emission by ruminants (Carulla *et al*. 2005; Puchala *et al*. 2005). Browse species with higher CT concentrations such as in *A. luederitzii* and *M. incanum* have good potential to reduce CH_4 concentrations as observed in previous studies (Getachew *et al*. 2008; Bhatta *et al*. 2009). Carulla *et al*. (2005) reported that when sheep were fed a mixture of *L. perenne* and *T. pratense* or when *M. sativa* was supplemented with 29 g CT/kg dietary DM of *A. mearnsii*, CH_4 emission was reduced by 130 kJ. However, a limited number of studies investigated the direct and indirect effects of plant secondary components on CH_4

Table 5. Loss of energy as methane (MJ/g DM) produced by browse species with different tannin concentrations supplemented to grass hay at a ratio of 30 : 70 with or without polyethylene glycol

Means with different lower-case letters within a column are significantly ($P < 0.05$) different. Means with different upper-case letters in rows within each incubation time are significantly ($P < 0.05$) different. No PEG, denotes presence of tannin; PEG denotes absence of tannin. CH_4, methane; IVOMD, *in vitro* organic matter digestibility; GE, gross energy

		GE (MJ/kg)	CH_4 (mL)		CH_4 (g/Kg)		Methane (g/Kg IVOMD)		GE loss as methane (MJ/Kg)	
Species	Tannin level		PEG	No PEG	PEG	No PEG	PEG	No PEG	PEG	No PEG
Acacia luederitzii	High	20.2	14.2	6.15	10.22	4.43	17.39	9.32	0.60bA	0.40cB
Monechma incanum	High	16.9	17.2	14.2	12.38	10.22	21.03	19.56	0.60bB	0.63bA
Acacia erioloba	Medium	19.1	13.33	10.88	9.59	7.83	19.07	16.25	0.72bA	0.64bB
Acacia haematoxylon	Medium	20.2	12.28	10.53	8.84	7.58	15.86	15.37	0.57cB	0.63cA
Olea europaea	Low	18.1	11.05	10.98	7.96	7.9	17.43	18.22	0.69bB	0.76bA
Acacia mellifera	Low	17.7	9.33	9.08	6.71	6.53	14.28	14.39	0.54cB	0.56cA
Eragrostis trichopophora	No tannin	11.4	18.78	17.63	13.52	12.69	24.7	23.33	1.07aA	1.16aA

production in animals, and it is difficult to provide a comprehensive assessment at this stage about the magnitude of decrease that might be realistically expected in *in vivo* research.

Loss of energy as CH_4

Grass-browse substrate containing high tannin browse species significantly decreased the amount of feed gross energy converted to CH_4 with *A. luederitzii* recording the least gross energy loss and *O. europaea* having the highest gross energy loss. According to Monforte-Briceño *et al*. (2005) and Tavendale *et al*. (2005), the action of CT on methanogenesis can be attributed to the indirect effects of reduced hydrogen production and organic matter digestibility, and by direct inhibitory effects on methanogens. Plant attributes that influence the amount of CH_4 produced in a sample are those chemical components that increase its fermentation potential, such as high crude protein, gross energy, organic matter digestibility values and low acid detergent lignin concentrations. In Table 5, CH_4 production was expressed as a ratio of gas volume, VFA and IVOMD with or without PEG and the ratios were significantly ($P < 0.001$) lower in substrates without PEG compared with the browse grass-substrates with PEG. The lower ratios for CH_4 : GP_{24}, CH_4 : VFA and CH_4 : IVOMD that were observed for *A. luederitzii* is partly due to *A. luederitzii* having higher fermentation properties compared with the other browse-grass substrates, but is also related to the tannin concentrations that reduced CH_4 production.

Conclusion

From this study, it can be concluded that the higher the tannin content of browse species, the greater their ability to reduce CH_4 concentration, though this correlated with a reduction in organic matter digestibility. *Acacia luederitzii* reduced CH_4 production by more than 55% over 48 h of incubation at the expense of 11% reduction in overall digestibility. Browse species with high tannin composition could therefore be used as a natural alternative to reduce CH_4 production. Further research may be required to determine at what concentration tannins can be supplemented to reduce CH_4 production without reducing digestibility or animal performance.

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References

Beauchemin KA, McGinn SM, Martinez TF, McAllister TA (2007) Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. Journal of Animal Science 85, 1990–1996.

Bhatta R, Vaidyanathan S, Shinde AK, Jackmola RC (2005) Effect of feeding complete feed block containing *Prosopis cineraria* leaves and polyethylene glycol (PEG)-6000 on nutrient intake, its utilization, rumen fermentation pattern and rumen enzyme profile in kids. *Journal of the Science of Food and Agriculture* **85**, 1788–1794.

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. *Journal of Dairy Science* **92**, 5512–5522.

Bodas R, Lopez S, Fernandez M, Garcia-Gonzalez R, Rodriguez AB, Wallace RJ, Gonzalez JS (2008) *In vitro* screening of the potential of numerous plant species as anti methanogenic feed additives for ruminants. *Animal Feed Science and Technology* **145**, 245–258.

Borris RP (1996) Natural products research: perspectives from a major pharmaceutical company. *Journal of Ethnopharmacology* **51**, 29–38.

Busquet M, Calsamiglia A, Ferret A, Cardozo PW, Kamel C (2005) Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. *Journal of Dairy Science* **88**, 2508–2516.

Callaway TR, Martin SA (1996) Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. *Journal of Animal Science* **74**, 1982–1989.

Carro MD, Ranilla MJ (2003) Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms *in vitro*. *British Journal of Nutrition* **90**, 617–623.

Carulla JE, Kreuzer M, Machmuller A, Hess HD (2005) Supplementation of *Acacia mearnsii* tannins decrease methanogenesis and urinary nitrogen in forage-fed sheep. *Crop and Pasture Science* **56**, 961–970.

Clapperton JL (1977) Effect of a methane-suppressing compound, trichloroethyl adipate, on rumen fermentation and growth of sheep. *Animal Production* **24**, 169–181.

Crutzen PJ (1995) The role of methane in atmospheric chemistry and climate. In 'Ruminant physiology: digestion, metabolism, growth and reproduction'. (Eds W Von Engelhardt, S Leonhard-Marek, G Breves, D Giesecke) pp. 291–316. (Ferdinand Enke Verlag: Stuttgart, Germany)

Engels EAN, Van der Merwe FJ (1967) Application of an *in vitro* technique to South African forages with special reference to the effect to certain factors on the results. *South African Journal of Agriculture Science* **10**, 983–995.

García-González R, Lopez S, Fernandez M, Bodas R, Gonzalez JS (2008) Screening the activity of plants and spices for decreasing ruminal methane production *in vitro*. *Animal Feed Science and*

Technology **147**, 36–52.

Getachew G, Pittroff W, Putnam DH, Dandekar A, Goyal S, DePeters EJ (2008) The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. *Animal Feed Science and Technology* **140**, 444–461.

Gibbs MJ, Lewis L, Hoffman JS (1989) 'Reducing methane emissions from livestock: opportunities and issues.' (U.S. Environmental Protection Agency: Washington, DC)

Goel G, Arvidsson K, Vlaeminck B, Bruggeman G, Descepper K, Fievez V (2009) Effects of capric acid on rumen methanogenesis and biohydrogenation on linoleic and á-linolenic acid. *Animal* **3**, 810–816.

Goering HK, Van Soest PJ (1970) Forage Fiber Analyses (Apparatus, reagents, procedures and some applications). USDA Agricultural Handbook No. 379. Agricultural Research Service, United States Department of Agriculture.

Grant RJ, Mertens DR (1992) Impact of *in vitro* fermentation techniques upon kinetics of fiber digestion. *Journal of Dairy Science* **75**, 1263–1272.

Guo Y, Liu Y, Lu Y, Zhu W, Denman S, McSweeney CS (2008) Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen micro-organisms. *Letters in Applied Microbiology* **47**, 421–426.

Hess HD, Monsalve LM, Lascano CE, Carulla JE, Diaz TE, Kreuzer M (2003) Supplementation of a tropical grass diet with forage legumes and *Sapindus saponaria* fruits: effects on *in vitro* ruminal nitrogen turnover and methanogenesis. *Australian Journal of Agricultural Research* **54**, 703–713.

Hess HD, Valencia FL, Monsalve LM, Lascano CE, Kreuzer M (2004) Effects of tannins in *Calliandra calothyrsus* and supplemental molasses on ruminal fermentation *in vitro*. *Journal of Animal and Feed Sciences* **13**, 95–98.

Hu WL, Liu JX, Ye JA, Wu YM, Guo YQ (2005) Effect of tea saponin on rumen fermentation *in vitro*. *Animal Feed Science and Technology* **120**, 333–339.

Johnson KA, Johnson DE (1995) Methane emissions from cattle. *Journal of Animal Science* **73**, 2483–2492.

Kamra DN, Agarwal N, Chaudhary LC (2006) Inhibition of ruminal methanogens by tropical plants containing secondary compounds. *International Congress Series* **1293**, 156–163.

Khiaosa-Ard R, Bryner SF, Scheeder MRL, Wettstein HR, Leiber F, Kreuzer M, Soliva CR (2009) Evidence for the inhibition of the terminal step of ruminal alphalinolenic acid biohydrogenation by condensed tannins. *Journal of Dairy Science* **92**, 177–188.

Lila ZA, Mohammed N, Kanda S, Kamada T, Itabashi M (2003) Effect of sarsaponin on ruminal fermentation with particular reference to methane production *in vitro*. *Journal of Dairy Science* **86**, 3330–3336.

Lourenco M, Cardozo PW, Calsamiglia S, Fievez V (2008) Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. *Journal of Animal Science* **86**, 3045–3053.

Martin C, Morgavi DP, Doreau M (2010) Methane mitigation in ruminants: from microbe to the farm scale. *Animal* **4**, 351–365.

McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA (2002) 'Animal nutrition.' (Pearson Education Ltd: Harlow, Essex)

Monforte-Briceño G, Sandoval-Castro CA, Ramirez-Aviles L, Capetillo Leal CM (2005) Defaunating capacity of tropical fodder trees: effects of polyethylene glycol and its relationship to *in vitro* gas production. *Animal Feed Science and Technology* **123–124**, 313–327.

Mould FL, Morgan R, Kliem KE, Krystallidou E (2005) A review and simplification of the *in vitro* incubation medium. *Animal Feed Science and Technology* **123–124**, 155–172.

Nagaraja TG, Newbold CJ, Van Nevel CJ, Demeyer DI (1997) Manipulation of ruminal fermentation. In 'The rumen microbial ecosystem'. (Eds PN Hobson, CS Stewart) pp. 523–632. (Springer: Dordrecht, The Netherlands)

Patra AK, Saxena J (2010) A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry* **71**, 1198–1222.

Patra AK, Kamra DN, Agarwal N (2006) Effect of plant extracts on *in vitro* methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. *Animal Feed Science and Technology* **128**, 276–291.

Pellikaan WF, Stringano E, Leenaars J, Bongers LJGM, Van Laar-Van Schuppen S, Plant J, Mueller-Harvey I (2011) Evaluating effects of tannins on extent and rate of *in vitro* gas and CH4 production using an automated pressure evaluation system (APES). *Animal Feed Science and Technology* **166–167**, 377–390.

Puchala R, Min BR, Goetsch AL, Sahlu T (2005) The effect of condensed tannin-containing forage on methane emission by goats. *Journal of Animal Science* **83**, 182–186.

Santoso B, Mwenya B, Sar C, Takahashi J (2007) Methane production and energy partition in sheep fed timothy hay silage- or hay-based diets. *Journal of Animal Science and Veterinary* **12**, 27–33. [JITV]

SAS (2010) 'SAS version 9.3.' (SAS Institute Inc.: Cary, NC)

Soliva CR, Zeleke AB, Clement C, Hess HD, 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. *Animal Feed Science and Technology* **147**, 53–71.

Tavendale MH, Meagher LP, Pacheco D, Walker N, Attwood GT, 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. *Animal Feed Science and Technology* **123–124**, 403–419.

Theodorou MK, Williams BA, Dhanoa MS, McAllen AB, France J (1994) A simple gas production method using pressure transducers to determine the fermentation kinetics of ruminant feed. *Animal Feed Science and Technology* **48**, 185–197.

Tilley JMA, Terry RA (1963) A two-stage technique for the *in vitro* digestion of forage crops. *Grass and Forage Science* **18**, 104–111.

Van Nevel CJ, Demeyer DI (1996) Control of rumen methanogenesis. *Environmental Monitoring and Assessment* **42**, 73–97.

Waghorn GC, Shelton ID (1997) Effect of condensed tannins in *Lotus corniculatus* on the nutritive value of pasture for sheep. *The Journal of Agricultural Science (Cambridge)* **128**, 365–372.

Waghorn GC, Shelton ID, McNabb WC (1994) Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 1. Non-nitrogenous aspects. *The Journal of Agricultural Science* **123**, 99–107.

Wang Y, McAllister TA, Yanke LJ, Xu ZJ, Cheeke PR, Cheng KJ (2000) *In vitro* effects of steroidal saponins from *Yucca schidigera* extract on rumen microbial protein synthesis and ruminal fermentation. *Journal of the Science of Food and Agriculture* **80**, 2114–2122.