Reduction in Methane Emissions from Ruminants by Plant Secondary Metabolites: Effects of Polyphenols and Saponins

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ABSTRACT

The effects of plant secondary metabolites (PSM), specifically polyphenols (tannins) and saponins on rumen fermentation and methanogenesis were investigated using the Hohenheim gas method. We evaluated the effects of: (1) polyphenol-containing plants, (2) simple phenols in the form of phenolic acids, (3) purified tannins, (4) saponin-containing plants, and (5) isolated saponin-rich fractions on rumen methanogenesis. Statistically significant negative relationships between total phenols, total tannins or tannin activity and methane (CH_4) production were observed, whereas no correlation existed between condensed tannins and CH_4 production. Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH_4 production significantly when added at 5 mM. Addition of purified chestnut and sumach tannins (hydrolysable tannins) at 1 mg/mL to the *in vitro* rumen fermentation system containing hay:concentrate (70:30) decreased CH_4 production ($P < 0.05$), by 6.5 and 7.2% respectively. However, addition of mimosa and quebracho tannins (condensed tannins) at this concentration did not decrease CH_4 production. For studying the effects of saponins, leaves of *Sesbania, Knautia* and seeds of Fenugreek, and their saponin-rich fractions were evaluated. Addition of Fenugreek and *Sesbania* plant materials to hay or the hay-concentrate mixture increased partitioning factor (PF, expressed as mg truly degraded substrate/mL gas produced; a measure of efficiency of microbial protein synthesis) and decreased CH_4 production per unit substrate degraded. These plant materials and their saponin-rich fractions did not reduce CH_4 production in absolute amounts despite decreases in protozoal numbers by 40–50%. The saponins altered the microbial community towards proliferation of fibre-degrading bacteria and inhibition of fungal population. The results with saponin-containing plant materials and their isolated fractions indicated a weak association between anti-protozoal activity of saponins and methanogenesis. Nevertheless, the saponin-containing plants possess potential to partition higher proportions of the substrate to microbial mass production

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Key words: *methane, polyphenols, saponins, microbial ecology, rumen fermentation.*

INTRODUCTION

The emission of greenhouse gases such as carbon dioxide and CH_4 is considered to be one of the most important global environmental issues (IPCC, 2001). Animals, particularly ruminants, produce CH_4 from anaerobic fermentation in their gastro-intestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. Ruminant livestock are responsible for about 15–20% of the total anthropogenic emission of CH_4 (Moss et al., 2000). The CH_4 produced from enteric fermentation of ruminants is not only related to environmental problems, but is also associated with energy losses and, hence reductions in their retention and use of energy. Typically 6–8%, but up to 12%, of the gross energy (GE) in feed is converted to CH_4 during microbial digestion in the rumen (Johnson and Johnson, 1995). Therefore, decreasing CH_4 production from ruminants is desirable for reducing greenhouse gas emissions and increasing utilisation of the digested energy. Plant secondary metabolites (PSM) have been suggested as effective alternatives to antibiotics to suppress rumen methanogenesis through their antimicrobial activity (Makkar et al., 2007; Jayanegara et al., 2009). Plant secondary metabolites constitute the group of chemicals present in plants that are not involved in the primary biochemical processes of plant growth and reproduction. The potential of these compounds and specifically of polyphenols (tannins) and saponins to reduce enteric CH_4 production has been recognised and extensive screening of a large range of plants and their secondary compounds is underway in several laboratories. The antimicrobial action and effects on rumen fermentation of these compounds depend on their nature, activity and concentration. We present here work conducted in our laboratory on the potential of various polyphenols (tannins), saponin-rich plants and isolated saponin-rich fractions to reduce CH_4 emission from ruminants.

RESULTS AND DISCUSSION

Polyphenols

The evaluation of polyphenols was conducted using the *in vitro* Hohenheim gas production method (Menke and Steingass, 1988) as modified by Makkar et al. (1995). We examined a number of polyphenol-containing plants, non-tannin simple phenolics, and purified tannins.

N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **151–157**

Table 1. Correlation coefficients between tannin assays and *in vitro* rumen methane production (n = 17).

Assays[b]	Methane (ml/100 mL)	Decrease in CH_4 (%)	Increase in CH_4[a] (%)
TP	–0.59*	0.57*	0.78***
TT	–0.60*	0.54*	0.62**
CT	–0.07[ns]	0.09[ns]	0.24[ns]
Tannin bioassay	–0.75***	0.79***	0.92***

TP = total phenols; TT = total tannins; CT = condensed tannins.
[a] Methane increase on polyethylene glycol (MW 6 000) addition; [b] for assay protocols see Makkar (2003a).
[ns] not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 2. Effect of addition of simple phenols on gas production, methane production and organic matter digestibility.

Treatment	Gas (mL)	CH_4 (mL/100 mL)	Decrease in CH_4 (%)	OMD (%)	CH_4/OMD (mL/100 mg)	Decrease in CH_4/OMD (%)
Control	76.2[c]	15.9[cd]	0.0[b]	76.1	5.05[bc]	0.0[ab]
Benzoic						
2 mM	77.0[c]	16.0[cd]	–0.6[ab]	75.2	5.19[c]	–2.8[a]
5 mM	74.8[bc]	16.0[cd]	–0.3[ab]	75.2	5.03[abc]	0.4[ab]
Cinnamic						
2 mM	75.3[bc]	15.5[abc]	2.6[bcd]	75.7	4.88[abc]	3.2[abcd]
5 mM	74.5[bc]	15.4[abc]	3.4[cde]	75.5	4.79[abc]	5.0[abcd]
Phenylacetic						
2 mM	73.3[abc]	15.9[bcd]	0.2[abc]	73.7	5.00[abc]	0.9[abc]
5 mM	74.3[bc]	16.4[d]	–3.1[a]	75.1	5.13[c]	–1.7[ab]
Caffeic						
2 mM	73.3[abc]	15.6[abc]	2.0[bcd]	73.2	4.94[abc]	2.1[abcd]
5 mM	71.0[ab]	14.9[a]	6.3[e]	73.4	4.57[a]	9.4[d]
p-Coumaric						
2 mM	72.5[abc]	15.5[abc]	2.4[bcd]	71.8	4.96[abc]	1.6[abcd]
5 mM	68.5[a]	15.1[a]	5.1[de]	71.0	4.61[ab]	8.5[cd]
Ferulic						
2 mM	72.5[abc]	15.9[bcd]	0.4[bc]	75.2	4.84[abc]	4.0[abcd]
5 mM	70.8[ab]	15.2[ab]	4.7[de]	71.4	4.77[abc]	5.5[bcd]
SEM	0.49	0.08	0.51	0.43	0.039	0.75

OMD = organic matter digestibility.
Values in the same column with different superscripts are different at P < 0.05.

Polyphenol-containing Plants

Using 17 polyphenol-containing plants (**Table 1**), statistically significant negative relationships between total phenols (TP), total tannins (TT) or tannin activity and CH_4 production existed, whereas the relationship between condensed tannins (CT) and CH_4 production was not significant. The highest correlation was found between tannin activity determined by the tannin bioassay and CH_4 decrease.

Since the correlations between TP and decrease in CH_4 or increase in CH_4 on addition of polyethylene glycol (a tannin-inactivating agent) were higher than those for TT, it seems that non-tannin phenols contribute to reducing CH_4 production. It would be interesting to obtain direct evidence by isolating non-tannin phenols and incubating them in the *in vitro* gas method. These results, if confirmed, could have wide application since non-tannin phenols are not likely to decrease the utilisation of proteins and other nutrients, but could also have beneficial effects (antioxidant, anticarcinogenic) associated with phenolic compounds (Makkar, 2003b; Makkar et al., 2007).

Although it was evident from these results that tannin-containing plants are able to reduce ruminal CH_4 emission, some reports suggest that tannins have no significant effect on rumen CH_4 production. For example, Oliveira et al. (2007) reported that there was no effect of tannin levels on CH_4 emission from diets containing sorghum silages. Beauchemin et al. (2007) also reported that feeding a diet containing an extract of quebracho tannins at a level up to 20 g/kg dry matter did not reduce enteric CH_4 emissions from growing cattle, although the protein-binding effect of the quebracho tannin extract was evident. The different results obtained using different tannins could be

attributed to their nature, structure or activity and to the concentrations at which they were used.

Non-tannin Phenolics

The above study indicated that non-tannin phenols play a role in CH_4 reduction. In the next study we evaluated six simple phenols (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids), as representatives of non-tannin phenols. All of these simple phenols were added at two different concentrations, i.e. 2 and 5 mM. The results are presented in **Table 2**.

In general, the addition of simple phenols decreased gas production although most of them were not significantly different and the effects were higher at higher concentrations. None of the simple phenols was effective in decreasing CH_4 production at the lower concentration (2 mM). Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH4 production significantly ($P < 0.05$) when added at 5 mM. Caffeic acid at 5 mM was the most effective of the simple phenols tested, decreasing CH_4 by 6.3% compared with the control. The magnitude was higher (9.4% compared with the control) when expressed as decrease of CH_4 per unit organic matter digested. After caffeic acid, the order of simple phenols to decrease CH_4 was: p-coumaric > ferulic > cinnamic. Phenolic acid containing tri-hydroxy group (caffeic acid) had a higher CH_4 inhibitory effect than those containing di-hydroxy groups (p-coumaric acid and ferulic acid). The phenolics containing a single hydroxy group (benzoic, phenylacetic and cinnamic acids) had the least effect. These results suggest that phenolics with higher numbers of hydroxyl groups are expected to elicit higher CH_4 inhibitory effects.

The effect of phenolic acids on methanogenesis could be expected since they affect the activities of rumen microbes. The decrease in ruminal CH_4 production could be linked to their role in inhibiting fibre degradation and in decreasing protozoa to a certain extent. Inhibition of fibre degradation will shift short chain fatty acid (SCFA) composition away from acetate and hence less production of hydrogen and less CH_4 formation. On the other hand, the anti-protozoal effect of phenolic acids would decrease CH_4 production since a portion of methanogens is attached to protozoa (Vogels et al., 1980). These protozoa-associated methanogens have been reported to contribute up to 37% of total rumen CH_4 emissions (Klieve and Hegarty, 1999). Therefore reduced protozoal counts in the rumen are associated with the reductions in CH_4 production, however, this is not always the case since a weak association between protozoal numbers and methanogenesis was observed with saponin-containing plants (discussed in later sections).

Phenolic acids are common constituents of forages fed to ruminants, where they occur most commonly as hydroxycinnamic acids ester-linked to polysaccharide. Ferulic and p-coumaric acids, the major phenolic acids found in this form, may represent up to 2.5% by weight of the cell walls of temperate grasses (Hartley and Jones, 1977). In the present study, phenolic acid concentrations of 2 and 5 mM were equal to 1.9–3.1 and 4.8–7.7% of the substrate dry matter incubated, respectively, depending on the structure and molecular weight of each phenolic acid. The lower concentration used was therefore in a reasonable range, while the higher concentration might also be in a reasonable range for the tropical forages, which generally contain higher concentrations of lignified tissues and secondary metabolites than temperate forages. In the *in vivo* situation, rumen microbes might encounter such a high concentration of phenolic acids provided that all phenolic acids are released from plant tissues, which is normally not the case. However, the microbes attached to the plant tissues are likely to encounter higher concentrations of phenolic acids in their microenvironment.

Purified Tannins

Some studies have reported that feeding tannin-containing forages to ruminants reduces CH_4 emissions (e.g. Puchala et al., 2005). However, in most of those studies, the reduction in CH_4 was confounded by changes in forage composition and quality. Lower fibre diets are associated with lower CH_4 emissions. Other nutrients such as lipid (oil) affect CH_4 production. Similarly, higher digestible feed is known to produce less CH_4 per unit feed intake. Negative effects on ruminal fibre digestion, which may relate to decreased number of cellulolytic bacteria, formation of tannin-cellulose complexes that are resistant to enzymatic digestion, and/or impaired substrate adhesion by fibrolytic microbes, would reduce hydrogen availability to lessen methanogenesis (Carulla et al., 2005). Thus, there is considerable uncertainty about the effectiveness of tannin-containing forages to reduce enteric CH_4 emissions from cattle. In the present study, therefore, other confounding components were omitted by using the purified tannins and, hence, specific effects of tannins were obtained. Different levels of purified tannins from chestnut, mimosa, quebracho and sumach (0.5, 0.75 and 1.0 mg/mL) were evaluated for their potential to reduce rumen CH_4 production. Chestnut and sumach tannins represented the hydrolysable tannins, whereas mimosa and quebracho tannins represented the condensed tannins.

The addition of purified chestnut and sumach tannins at 1 mg/ml to a hay:concentrate (70:30) diet significantly decreased ($P < 0.05$) CH_4 production by 6.5 and 7.2% respectively. Lower concentrations (0.5 and 0.75 mg/mL) did not significantly decrease CH_4 production. The addition of mimosa and quebracho tannins (condensed tannins) did not significantly decrease CH_4 production, even at the highest concentration. For all tannins, increases in concentration led to increases in CH_4 reduction (**Figure 1**). The condensed tannins decreased gas production and organic matter digestibility (OMD) more than the hydrolysable tannins. The results suggested that the hydrolysable tannins are more effective in decreasing CH_4 emissions than the condensed tannins, while at the same time the hydrolysable tannins did not significantly decrease OMD. The condensed tannins appear to decrease CH_4 more through reduced fibre digestion (indirect effect), while hydrolysable tannins act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The tannin concentrations of 0.5, 0.75 and 1.0 mg/mL were equal to 4.0, 5.9 and 7.9% of the substrate dry matter, respectively. Animals are likely to encounter such concentrations, especially in tropical regions, where they are exposed to high amount of tannins during the dry season. During the dry season, animals depend largely on fodder tree leaves and browses, and the tannin content in these feed resources is generally high (5–15%). However, it may be noted that the effects on the extent of CH4 reduction of tannins in the soluble form as in this study and in *in vivo* situations where tannins are a part of the feed might be different. Nevertheless, the *in vitro* studies give insight into the mechanism of action of various tannins, their comparative effects and possible *in vivo* effects.

Although it was evident from our research that polyphenols reduce rumen CH_4 production significantly, it should be noted that we used *in vitro* experiments to measure the effects. Flachowsky and Lebzien (2009) noted that it is extremely difficult to extrapolate from *in vitro* measurements to *in vivo* situations in ruminants, or to field conditions. This is because the relationship between CH_4 produced *in vivo* and *in vitro* is very poor ($r^2 = 0.264$). However, the *in vitro* studies should be considered as a starting point for screening of potential CH_4 inhibitors and should be combined with *in vivo* experiments. Therefore, Flachowsky and Lebzien (2009) proposed a three-step programme to assess the CH_4-reduction potential of feed additives or feeding measurements. This includes *in vitro* screening of substances,

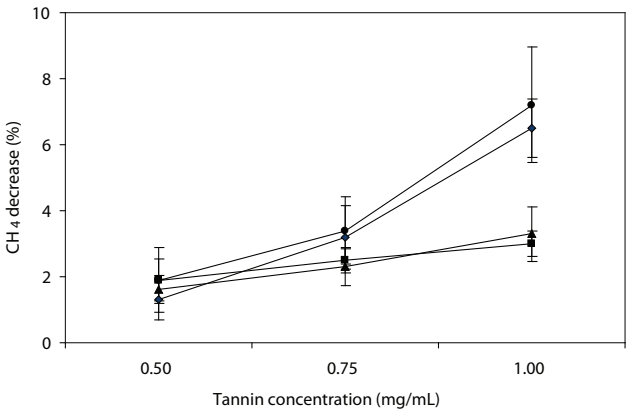

Figure 1. Effect of purified tannins from chestnut (-♦-), mimosa (-■-), quebracho (-▲-) and sumach (-●-) on CH_4 decrease when added to hay:concentrate diet (70:30 w/w) at concentrations of 0.5, 0.75 and 1.0 mg/mL

short-term *in vivo* experiments in target animals and finally *in vivo* long-term recording of CH_4 production together with other animal performance parameters. Following this programme will substantially increase the relevance of such studies to the industry and potential users. Also, short term *in vivo* studies could also be replaced by continuous fermentation studies as conducted by Goel et al. (2009). This would save time and resources.

Saponins

Saponins are natural detergents, chemically defined as high molecular weight glycosides in which sugars are linked to a triterpene or steroidal aglycone moiety. These compounds possess membranolytic properties, resulting in cell death by forming complexes with sterols on protozoal cell membranes (Cheeke, 1999). They modify ruminal fermentation by suppressing rumen protozoa and selectively inhibiting some bacteria. The symbiosis of protozoa with methanogenic bacteria in the rumen is well established and selective suppression of protozoa has been suggested to be a promising approach to reduce the CH_4 production (Dohme et al., 1999). Therefore, the plants rich in saponins have potential for enhancing flow of microbial protein from the rumen, increasing the efficiency of feed utilisation, and decreasing methanogenesis. We studied the effects of saponin-containing plants and their saponin-rich fractions for their anti-protozoal and CH_4 inhibition activities using the Hohenheim Gas Test (HGT). The first study was designed to observe the effect of saponin-rich plant materials on partitioning of nutrients from roughage- and concentrate-based feeds to CH_4, followed by another similar study with their isolated saponin-rich fractions. Fermentation parameters and microbial community structure were also investigated.

Saponin-containing Plants

The incubation of saponin-containing plant materials: leaves from *Sesbania* (*Sesbania sesban*) or seeds of Fenugreek (*Trigonella foenum-graecum* L.) as a sole substrate resulted in higher responses towards increasing the partitioning factors, PF (increasing efficiency of microbial mass synthesis) and increasing the reductions in CH_4 production (**Table 3a**). The plant materials when added to hay- or concentrate-based diets, did not produce substantial reductions in CH_4 production (**Table 3b**). The higher PF and CH_4 reductions were observed when the saponin-containing plant materials were added to concentrate-based diets. The crude plant extracts (in water and methanol/water (0.95:0.05, v/v) of the test plants had negligible effects on CH_4 production (data not shown). All different incubation materials: the sole plant material, plant material supplemented with hay- or concentrate-based diets or the plant extracts resulted in reductions in protozoal populations. However, these reductions did

Table 3. Effect of different plant materials on methane production.

a) Incubation with sole plant materials as substrate (380 mg/40 mL incubation fluid).

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate-hay fed animal				
Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)
Hay	3.11a				Conc	3.16a			
Fenugreek	3.35a	-2.2	6.7a	68.1	Fenugreek	4.57b	20.5 b	29.1 a	73.2 b
Sesbania	4.63b	-3.4	30.4b	61.1	*Sesbania*	3.52a	5.4a	37.8 b	66.0 a
SEM	0.051	1.22	0.37	1.26	SEM	0.14	1.93	0.11	2.12

b) Supplementation of hay or concentrate (380 mg each) with the supplement (66 mg)

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate - hay fed animal				
Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)
Hay	3.11^{a}				Conc	3.16^{a}			
Hay+F	3.28^{a}	0.56	5.18^{a}	49.5	Conc+F	3.33^{ab}	4.86^{b}	9.74^{a}	56.0
Hay+S	3.45^{a}	1.13	11.4^{b}	47.8	Conc+S	3.52^{b}	1.62^{a}	11.9^{ab}	50.7
SEM	0.096	0.124	2.028	0.98	SEM	0.132	0.221	1.948	1.03

PF = partition factor (mg of substrate truly degraded/mL gas); MR = methane reduction on volume basis; MR_{TD} = methane reduction on the basis of substrate truly degraded; F = Fenugreek seeds and S = *Sesbania* leaves.

Values in the same column with different superscripts are different at $P < 0.05$.

not accompany the decreases in CH_4 production in the incubations using rumen liquor from hay-fed animals; on the other hand, small reductions in CH_4 were produced in incubations with rumen liquor from concentrate-fed animals ($P < 0.05$). This observation indicates a weak association between the two parameters. The results from this study imply that the supplementations tested did not adversely affect the degradability of the basal feeds, hay or concentrate-hay mixture. Nevertheless, these plant materials when used as supplements, especially to the concentrate-based diet, have the potential to partition higher proportions of the substrate to microbial mass production and to elicit some CH_4 reduction per unit of substrate degraded. A weak association between protozoal number and methanogenesis was evident in this study and this association seemed to be diet dependent.

Saponin-rich fractions

Based on the results from saponin-containing plants, a further study was conducted with saponin-rich fractions prepared from the test plant materials: leaves of *Sesbania* (*Sesbania sesban*), *Knautia* (*Knautia arvensis*) and seeds of Fenugreek (*Trigonella foenum-graecum* L.). These fractions were evaluated for their effects on partitioning of nutrients from the concentrate-based diets to CH_4, SCFA and protozoa number. Additionally, changes in ammonia nitrogen (an important parameter in determining the N flow during substrate degradation which is used for microbial biomass production and absorption through the rumen cell wall), ammonia uptake and microbial community structure were also studied using real-time PCR assay (Denman and McSweeney, 2006). The lower concentration of saponin-rich fractions used in this study corresponded to the quantity of raw material of the test supplements which showed promising response in the former study, except for *Knautia* leaves, which were not evaluated in the earlier study. Saponins have been reported to alter rumen fermentation by affecting the digestibility (either increase or no effect) and increasing microbial protein synthesis (Makkar et al., 1998). In the present study the fractions did not affect digestibility, and a trend towards slightly higher gas production was observed, which might be due to the saponin-mediated increase in fiber degrading bacteria (discussed below). Increase in the PF was not observed on supplementation of any of the saponin-rich fractions (**Table 4**), while increased PF values were observed on supplementation of the plant materials from which these saponins were isolated. Different responses have been observed for different saponins, which could be attributed to the different nature and/or concentration of saponins. Therefore, caution should be exercised in generalising the effects of saponins.

The maximum CH_4 reduction was observed for higher concentrations of saponin-rich fractions of *Sesbania* (6.1%) and *Knautia* (6.4%). Saponin-rich fractions were not as effective as the original plant material which when used as equivalent to the lower concentrations of saponin-rich fractions from Fenugreek seeds and *Sesbania* leaves decreased the protozoal count by nearly 50%, while this inhibition was 39% and 36% only by the corresponding saponin-

Table 4. Effect of saponin-rich fractions of test plants on methane production and protozoal numbers.

Substrate*	Partition Factor	MR%	MR_{TD} (%)	Protozoa** ($\times 10^4$ mL/1)
S	3.25			19.54
S+F 5.62	3.12	1.82	–1.59	16.60 (15)
S+F 11.54	3.07	1.97	–4.47	11.93 (39)
S+Se 10.9	3.14	4.69	1.54	16.80 (14)
S+Se 21.8	3.08	6.14	1.71	12.41 (36)
S+K 3.88	3.16	5.50	3.23	16.83 (14)
S+K 7.76	3.16	6.43	3.94	14.66 (25)
SEM	0.122	1.821	1.112	1.224

*S = hay:concentrate (1:1), saponin-rich fractions (in mg) from: F = fenugreek; Se = *Sesbania*; K = *Knautia*. **Values in parentheses are the percentage reduction in protozoal number.

M — methane; MR — methane reduction on volume basis; TD — truly degraded substrate; MR_{TD} — methane reduction on truly degraded substrate basis.

Table 5. Effects of saponin-rich fractions on SCFA and ammonia uptake.

Substrate*	Total SCFA (µmol m/L)	C2:C3	Branched SCFA (µmol m/L)	NH_3–Nitrogen (mg m/L)	NH3-uptake (mg NH_3 m/L gas)
S	871.6	3.63	15.61	0.37	0.0747
S+F 5.62	1014.0	3.76	17.79	0.37	ND
S+F 11.54	837.2	3.39	17.12	0.35	0.0934
S+Se 10.9	849.1	3.76	12.12	0.36	ND
S+Se 21.8	911.9	3.56	14.30	0.36	0.0618
S+K 3.88	866.3	3.60	13.76	0.37	ND
S+K 7.76	1035.2	3.77	11.82	0.37	ND
SEM	10.11	0.045	1.234	0.056	

* — as in Table 1; SCFA C2:C3 — acetate:propionate; ** — iso-butyrate + iso-valerate; ND — not determined.

rich fractions, as observed in the present study. This reduction in the activity of saponin-rich fractions could be due to non-extraction of all the saponins or to a change of saponin activity during their extraction.

No difference was observed in the SCFA production amongst control and saponin-containing incubations (**Table 5**). A slight decrease in ammonia concentration was observed only with Fenugreek (11.54 mg/ 40 mL) and *Sesbania* (10.9 and 21.8 mg/40 mL buffer) saponin-rich fractions. Wide variations of the effects of saponins on ammonia concentration have been reported in the literature. In a review by Wina et al. (2005), 14 reports indicate no effect of saponins on ammonia while another 17 studies state a negative correlation between saponin and ammonia production. The slight decrease in ammonia concentration might be due to high anti-protozoal activities of Fenugreek and *Sesbania* saponins at their higher concentrations. The lower number of protozoa results in lesser bacterial lysis and therefore lower release of breakdown products of protein. The rate of NH_3-N uptake (an index of the efficiency of microbial protein synthesis) was calculated as the slope of a linear regression between the amount of NH_3-N (in mg) and net gas (mL) (Getachew et al., 1998). The higher slope values on supplementation of saponin-rich fraction from Fenugreek (**Table 5**) suggest increased ammonia uptake by rumen microbes which means that the nitrogen from feed is converted into microbial protein to a greater extent in the presence of these saponins. But this increase in microbial protein was not reflected in the PF values, probably due to the measurement of PF at an inappropriate time (24 h in this study) and erosion of PF differences by microbial lysis (Blümmel et al., 2003).

Saponin-rich fractions changed the microbial population as estimated by the comparative delta Ct method (Denman and McSweeney, 2006). *Sesbania* saponins decreased methanogen population by 78%. Reduced rumen fungal populations (20–60%) and increases in *Fibrobacter succinogenes* (21–45%) and *Ruminococcus flavefaciens* (23–40%) were observed (**Figure 2**). In absolute terms, increases were observed in total bacterial population as indicated by decreased threshold cycle (Ct) values on supplementation of saponin-rich fractions. This effect was expected due to decrease in protozoal numbers since there is no predation of bacteria by protozoa (Matheiu et al., 1996). The increased populations of *F. scuccinogenes* can be attributed to their resistance to saponins as observed by Wang et al. (2000), suggesting that this species has the ability to deglycosylate and therefore inactivate saponins. Vinogradov et al. (2001) also reported that the presence of 2-aminoethylphosphoric acid and glycolipid in the membrane enhances the membrane stability of *F. succinogenes*. Saponin-rich fractions were inhibitory to ruminal fungi as well. The inhibition of protozoal population also resulted in inhibition of methanogens though the effect was more pronounced for saponin-rich fraction of *Sesbania*. The Fenugreek fraction being more inhibitory to protozoa did not result in higher suppression of methanogens, which again reconfirms the weak association between the protozoal population and methanogen numbers.

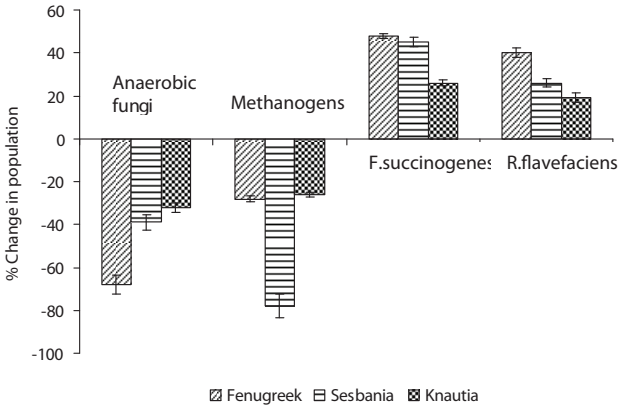

Figure 2. Effect of saponin-rich fractions on percent changes in microbial population.

Results for the effects of saponin-rich fractions on methanogens and CH4 levels were unexpected. These fractions decreased protozoa numbers and methanogen populations but did not decrease CH_4 production. The association between methanogens and protozoa is not obligatory and there is considerable evidence that different groups of methanogens are not equally associated with ciliate protozoa. A weak relationship between methanogenesis and the methanogen population expressed as a proportion of total anaerobes was observed by Nollett et al. (1998) under both *in vitro* and *in vivo* conditions. In our study, the lack of inhibition of CH_4 production with decreases in methanogens could have been caused by (i) a slow rate of association between protozoa and methanogens due to higher generation time of protozoa as compared with methanogens, (ii) an increased metabolism of methanogenic microbes independent of species remained after addition of saponins, and/or (iii) by changes in composition of the methanogenic community (Machmüller et al., 2003) and their increased efficiency of CH_4 production. The two major groups of methanogens in rumen: methanobacteriaceae (99.1% of total archea associated with protozoa) and free living methanobacteriales (0.05%) differ in their physiological characteristics. Therefore, based on the present results, it is suggested that on inhibition of protozoa, the species belonging to methanobacteriaceae declined with an increase in the number of free-living methanobacteriales. The reduced rate of association of protozoa and methanogens could result in higher interspecies hydrogen transfer between increased population of both hydrogen producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living methanobacteriales indicating no effect on CH_4 production.

CONCLUSIONS

Polyphenol or tannin containing plants decreased CH_4 production and, therefore, could be strategically used in diets for decreasing CH_4 emissions from ruminants. Amongst the tannin assays, tannin bioassay (a reflection of tannin activity) was the best predictor of the CH_4 reduction potential of a plant. Total phenols and total tannins were also good predictors of the CH_4 reduction potential. Methane decrease by addition of phenolic acids was relatively small (up to 6.3%), and the effect of phenolic acids on CH_4 reduction depended on the source and concentration applied. The order of simple phenols to decrease CH_4 was: caffeic acid > p-coumaric > ferulic > cinnamic. Hydrolysable tannins had greater ability to decrease CH_4 production and CH_4 production per unit organic matter digested than condensed tannins. The condensed tannins appear to decrease CH_4 more through a reduction in fibre digestion (indirect effect), while hydrolysable tannins appear to act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The saponin-containing plants did not produce substantial reductions in CH_4 production but showed the potential to partition higher proportions of the substrate to production of microbial mass. The saponins tested possessed anti-protozoal activity but did not result in CH_4 inhibition suggesting that the uni-directional relationship between protozoal numbers and methanogenesis, as affected by

saponins, is not obligatory. However, the inhibition of methanogen population led to increases in fibre-degrading bacterial groups.

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