Animal Science

REGULAR ARTICLE

Effect of *Camellia sinensis* and *Trigonella foenum-greacum* saponins on *in vitro* rumen fermentation of vetch-oat hay

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Abstract

The present study was conducted to investigate the effect of two plants rich in saponins on *in vitro* ruminal fermentation traits of vetch-oat hay using gas syringes as incubators. Two plants, *Camellia sinensis* and *Trigonella foenum-greacum,* were added to 200 mg of vetch-oat hay at levels of 0, 2, 4, 6 and 8 mg and 0, 48, 54, 60 and 66 mg, respectively. Gas production was dose-dependent for both plants and decreased for all incubation times with the increasing doses. Methane concentration was decreased at all inclusion levels and for each incubation time. The highest methane reduction was observed at 48h of incubation for both plants. This decrease varied between 48.78-52.84% and 45.52-72.35% for *Camellia sinensis* and *Trigonella foenum-greacum*, respectively. Ammonia-N concentrations also decreased significantly (P < 0.002) when the plants rich in saponins were included with the vetch-oat hay. In addition, these plants significantly inhibited the protozoa growth in ruminal fluid (P < 0.001). At 24h incubation, protozoa counts were reduced by 81.86% and 83.29% for the high levels of *Camellia sinensis* and *Trigonella foenum-greacum*, respectively. Finally, *in vitro* truly dry matter digestibility was significantly affected by the inclusion of plants rich in saponins (P < 0.05). It is suggested that addition of these saponin-rich plants to feed could modify the rumen fermentation and inhibit the release of methane and ammonia, which may be beneficial for improving nutrient utilization and animal growth.

Key words: Ammonia-N, Digestibility, Fenugreek, Gas production, Methane, Protozoa

Introduction

Greenhouse gas emissions from livestock are of increased worldwide concern. These emissions, predominantly of methane and nitrous oxide, are major contributors to their respective anthropogenic inventories, and with livestock populations growing to meet an increasing global demand for food, emissions will increase concomitantly (Lassey, 2007; Ramírez-Restrepo et al., 2010). Enteric methane contributes 30-40% of total methane production from agricultural sources (Moss et al., 2000). Furthermore, methane is a particularly potent gas as it has 25 times more global warming potential than carbon dioxide (Francis et al., 2002) and its half-life in the atmosphere is estimated

to be 12 years compared to that of carbon dioxide which is estimated to be a century (Agarwal et al., 2009). In addition, the excretion of methane from the rumen can represent a loss of 0.15 of the digestible energy, depending on the type of diet (Goel et al., 2008). For this reason, many attempts such as concentrate supplementation (Lovett et al., 2005), use of probiotics and prebiotics (Mwenya et al., 2004; Takahashi et al., 2005), lipid supplementation (Ungerfeld et al., 2005), and the addition of plant extracts (Makkar, 2005; Patra et al., 2006; Goel et al., 2008) have been made to decrease enteric methane production. Antibiotic growth promoters also decrease methane production (Fuller and Johnson, 1981). However, use of these products has been banned in Europe since 2006 and many countries outside the European Union are also considering a ban, *i.e*, Algeria. As a result, researchers have intensified efforts to exploit plants, plant extracts and natural plant compounds as potential natural alternatives for enhancement of livestock productivity while minimizing environment impacts (Makkar et al., 2007), including decreased methane production

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(Soliva et al., 2008). Among these, inclusion of saponin containing plants has received wide interest since saponins are known to lyse protozoa and decrease rumen protozoal counts and methanogenesis (Hart et al., 2008). Therefore, the present study was conducted to evaluate the impact of two plants rich in saponins, tea (*Camellia sinensis*) and fenugreek (*Trigonella foenum-graecum*), incubated in association with vetch-oat hay on in vitro fermentation, protozoa count and methane production.

Material and methods
Materials

The substrate used in the incubation was vetch-oat hay (DM, 891.1 g/kg; ash, 50.8 g/kg DM; CP, 67.9 g/kg DM; NDF, 616.2 g/kg DM; ADF, 327.7 g/kg DM; ADL, 43.6 g/kg DM), sun-dried and milled to pass 1-mm sieve. Tea (*Camellia sinensis*, DM; 893.3g/kg, ash; 133.3 g/kg DM, total sugars; 541.8g/kg DM, saponins; 180g/kg DM) and fenugreek (*Trigonella foenum-graecum*, DM; 913.3 g/kg, ash; 144.7 g/kg DM, total sugars; 308.6 g/kg DM, saponins; 300 g/kg DM) were obtained from SONAS-I.U.T. laboratory, Angers University, France.

Experimental design

Calibrated propylene syringes were used as incubators. The additives were added to 200 mg of vetch-oat hay in ruminal fluid at levels of 2, 4, 6, 8 mg and 48, 54, 60, 66 mg for tea and fenugreek, respectively. Triplicate syringes were used for each sampling. In the same way, three syringes without additives (control, vetch-oat hay with inoculum) and three others containing only inoculum (blank, rumen juice and artificial saliva) were incubated under the same conditions. Syringes were incubated at 39°C in an isothermal incubator equipped with a rotor, which ran continuously at 9 rpm. Gas production (GP) was recorded at 3, 6, 24, 48, 72 and 96h of incubation. Methane production was noted after 3, 24, 48 and 96h of incubation. After 24h, the inoculants were analysed for pH, ammonia-N, protozoa count and *in vitro* true digestibility of dry matter (IVTDMD).

In vitro gas production studies

The GP was determined according to Menke et al. (1979). About 200 mg of substrate were accurately weighed and put into 60 propylene syringes fitted with plungers. Syringes were filled with 30 ml of culture medium consisting of 10 ml rumen fluid and 20 ml buffer solution prepared as described by Menke and Steingass (1988). Rumen fluid was obtained from three healthy slaughtered sheep that were maintained on pasture. The rumen

fluid was filtered through four layers cheesecloth and brought to the laboratory in pre-warmed Thermos flasks.

Measurements of in vitro fermentation parameters
Fermentation parameters

After 24h of incubation, each batch of medium was checked for pH using a calibrated pH meter (Hanna 2039). Methane (CH_4) was measured chemically by transferring the total gas into syringes containing 4 ml of sodium hydroxide (NaOH, 10N) which absorbs carbon dioxide, the gas remaining was assumed to be CH_4 (Jouany 1982). Ammonia-N ($N-NH_3$) concentration was determined by the phenol–hypochlorite method using spectrophotometric determination as described by Chaney and Marbach (1962). Two ml of orthophosphoric acid (50 g/l) were mixed into 10 ml of syringe content. Samples were then centrifuged at 15000g for 15 min at 4°C and the supernatant was used to determine ammonia concentration. The ammonia of blanks was subtracted from the measured $N-NH_3$ of samples to obtain the net $N-NH_3$ concentration.

In vitro true digestibility determination

After 24h of incubation, the contents of the syringes were digested with neutral detergent solution (NDS solution) and the undigested feed was recovered on crucibles N°C, washed and dried at approximately 90°C for 16h. The substrate truly degraded in 24h was calculated by subtracting the DM of undigested feed from the DM incubated in the syringe (Blümmel et al., 1997).

Protozoa count

The methodology described by Ogimoto and Imai (1981) for protozoa count was used. After 24h of incubation, 100 µl of the syringe contents was mixed with an equal volume of the methylgreen-formaldehyde-saline fixative solution containing methylgreen (0.06 g/100 ml), sodium chloride (0.8 g/100 ml) and 1:10 diluted formalin (35% w/v, HCHO in water). The mixture was shaken gently, held in dark for 30 min and then introduced into a Malassez chamber. The protozoa were then counted using microscopy at 40 magnifications.

Calculation and statistical analysis

The rate and extent of gas production were calculated by non-linear regression using an exponential model $Y = a + b\,(1-e^{-k*t})$, where Y is gas volume at time t, $(a +b)$ is potential gas production and k is rate at which gas is produced (Orskov and McDonald, 1979).

The data from the two additives were analysed separately using two way ANOVA (sample and dose) option in the SAS/STAT software (Statistical Analysis System, 1990). The means were compared by the Scott-Knott's test at the level of 5%, using the entirely random design.

Results

Effects of tea and fenugreek inclusion on in the vitro gas and methane productions

The *in vitro* GP recorded after 96h of incubation and exponential parameters are presented in Table 1. For all increasing levels of tea and fenugreek, total GP was significantly decreased at all incubation times compared to control (P < 0.05). This reduction in total GP is dose-dependent. At 24h of incubation, GP reduction ranged between 3.61-21.9% and 7.48-26.45% for tea and fenugreek, respectively. The addition of tea and fenugreek positively influenced the parameters of the exponential model. For both plants, GP from the insoluble fraction (*b*) was increased except for the

lowest dose (2 mg) for tea and the highest level (66 mg) for fenugreek. In the same way, the rate of GP from the insoluble fraction (*k*) significantly increased compared to the control (P < 0.05). However, GP from the soluble fraction significantly decreased compared to the control (P < 0.05).

For both plants, methane production was decreased at all doses and at all incubation times compared to the control (Figure 1). This indicates that methane production began to decline at 3h of incubation, reached the lowest level at 48h of incubation and then ascended gradually. At 24h of incubation, the addition of 2, 4, 6 and 8 mg of tea reduced methane production by 48.78, 49.18 and 52.84%, respectively. When fenugreek was included at 48, 54, 60 and 66 mg, methane concentration was decreased by 45.52, 34.96, 72.35 and 67.1%, respectively, suggesting that PRS are good natural promoters for methane inhibition.

Table 1. Effects of the different levels of tea and fenugreek inclusion on *in vitro* gas production (ml) and exponential model parameters.

| Adding levels of tea									
0 (mg)	3h	6h	24h	48h	72h	96h	*a* (ml)	*b* (ml)	*k* (%.h^{-1})
	10.67[a]	18.00[a]	27.67[a]	34.77[a]	37.67[a]	38.00[a]	8.52[a]	29.93	5[d]
2 (mg)	9.33[b]	15.00[a]	26.67[b]	28.67[b]	25.67[b]	24.43[b]	-1[b]	27.49	26.67[a]
4 (mg)	7.47[c]	13.67[c]	26.17[b]	28.07[b]	24.67[c]	22.33[c]	-4.32[c]	29.72	26.17[a]
6 (mg)	6.00[d]	14.13[c]	22.67[c]	24.33[c]	23.67[d]	20.56[d]	-9.79[d]	32.64	22.67[b]
8 (mg)	5.57[d]	12.67[d]	21.61[d]	22.87[d]	20.91[e]	18.93[e]	-8.19[d]	29.31	21.61[c]
S.E.M.	0.62	0.41	0.51	0.49	0.45	0.39	1.67	1.76	2
Pr.	0.01	0.05	0.003	0.001	0.002	0.001	0.001	0.056	0.005
Adding levels of fenugreek									
0 (mg)	3h	6h	24h	48h	72h	96h	*a* (ml)	*b* (ml)	*k* (%.h^{-1})
	10.67[a]	18.00[a]	27.67[a]	34.77[a]	37.67[a]	38.00[a]	8.52[a]	29.93[d]	5
48 (mg)	8.00[b]	16.67[b]	25.60[b]	30.33[b]	28.67[b]	27.59[b]	-3.58[c]	31.97[c]	16
54 (mg)	7.5[b]	16.77[b]	24.40[b]	29.22[c]	28.04[b]	26.67[c]	-7.98[d]	35.44[a]	11.5
60 (mg)	5.93[c]	14.96[c]	23.95[c]	27.07[d]	25.44[c]	25.05[d]	-9.61[e]	34.96[b]	12.2
66 (mg)	5.17[c]	13.26[d]	20.35[d]	26.00[e]	24.46[d]	23.00[e]	3.50[b]	27.46[e]	14.5
S.EM.	0.54	0.37	0.42	0.44	0.37	0.09	2.65	0.80	2.8
Pr.	0.001	0.003	0.001	0.002	0.001	0.001	0.01	0.02	0.16
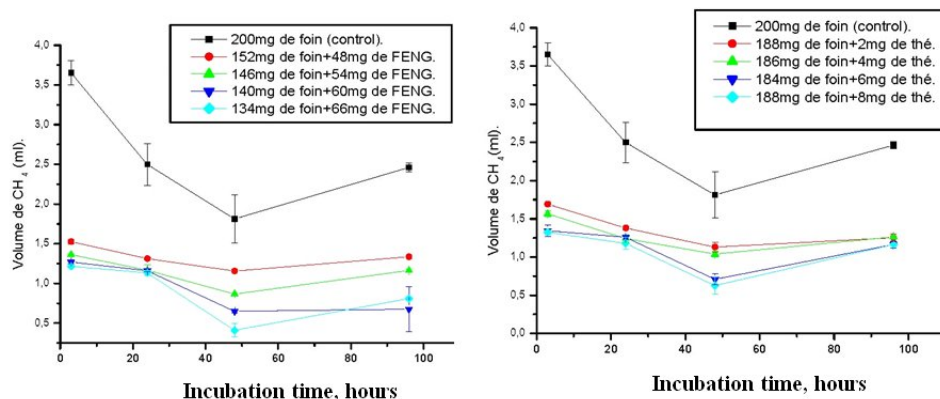
Figure 1. Effects of tea and fenugreek addition on *in vitro* methane production at different incubation times. Bars indicate standard error. Where not visible, bars fall within symbols.

Table 2. Effects of tea and fenugreek inclusion on in vitro methane production and rumen fermentation parameters recorded after 24h of incubation.

Adding levels of tea					
0 (mg)	Methane reduction (%)	pH	Ammonia-N (10^2 mg/l)	IVTDMD (%)	Protozoa (10^3 cell/ml)
	100	6,58	41,7[a]	58,17[a]	4,19[a]
2 (mg)	48.78	6,52	31,33[b]	48,67[b]	2,46[b]
4 (mg)	48.78	6,74	27,07[c]	45,17[b]	2,21[b]
6 (mg)	49.18	6,71	26,09[c]	44[c]	1,96[c]
8 (mg)	52.84	6,62	23,80[d]	43,77[c]	0,76[d]
S.E.M.		0,28	3,11	3,26	0,11
Pr.		0,13	0,003	0,042	0,01
Adding levels of fenugreek					
0 (mg)	Methane reduction (%)	pH	Ammonia-N (10^2 mg/l)	IVTDM (%)	Protozoa (10^3 cellules/ml)
	100	6,58	41,70[a]	58,17[a]	4,19[a]
48 (mg)	45.52	6,78	32,37[b]	43,63[b]	2,40[b]
54 (mg)	34.96	6,59	28,77[b]	42,30[b]	2,16[c]
60 (mg)	72.35	6,53	23,33[b]	39,28[c]	1,84[d]
66 (mg)	67.1	6,66	12,50[c]	38,82[d]	0,70[e]
S.EM.		0,18	4,21	0,96	0,16
Pr.		0,20	0,002	0,001	0,001

Effects of tea and fenugreek addition on in vitro fermentation parameters

The impact of both these PRS on *in vitro* fermentation parameters is illustrated in Table 2. It reveals that inclusion of tea and fenugreek did not affect pH values recorded at 24h of incubation (P>0.05). All values noted were in the normal range for good cellulolytic activity. The addition of both PRS significantly decreased N-NH$_3$ concentration (P<0.001). This decrease was dose-dependent. At 24h of incubation, N-NH$_3$ concentration was decreased by 24.86, 35.1, 37.43 and 49.47% for 2, 4, 6 and 8 mg of tea, respectively. A similar trend was also observed for the fenugreek addition, where ruminal N-NH3 concentration was reduced by 22.37, 31, 44.05 and 72.02% for 48, 54, 60 and

66 mg, respectively. Otherwise, addition of tea and fenugreek reduced significantly *in vitro* truly dry matter digestibility (IVTDMD) (P < 0.05). This parameter decreased linearly with increasing levels of tea and fenugreek (P < 0.01). The addition of both PRS also inhibited the ciliate protozoa growth in the culture media. After 24h of incubation, the decrease in protozoa count was 41.28, 47.25, 53.22 and 81.86% for 2, 4, 6, 8 mg of tea, respectively. For fenugreek, its anti-protozoa activity was slightly higher than that of tea. For 48, 54, 60 and 66 mg of fenugreek inclusion, the decrease in ruminal fauna was 42.72, 48.44, 56.08 and 96.18%, respectively.

Discussion

Methane is produced as a by-product of the ruminal fermentation process. It represents not only an important loss of feed energy for the ruminants, but also a potent environmental pollutant. Therefore, minimising methane excretion by ruminants could have important economical and environmental benefits. In the present study, the addition of tea and fenugreek significantly reduced both GP and methane concentrations. The impact of these PRS on methane concentration was greater than on total GP. These results are in agreement with previous studies also using PRS (Hess et al., 2003; Pen et al., 2006; Goel et al., 2008; Holtshausen et al., 2009). While, Hue et al. (2005) noted that GP was enhanced by tea saponins, especially in the early stage of fermentation. Whereas, Pen et al. (2006) observed that *Quillaja saponaria* extracts did not affect GP or and methane production. This variability is undoubtedly explained by differences in saponin type and concentration as well as the nature and composition of the fermentation substrate may influence the response to saponins supplementation. Methane production in the rumen involves both *Archae bacteria* (methanogens) and protozoa. Based on this, the antimethanogenic activity of saponins was presumably a direct action against these microorganisms. The role of protozoa in methane production is primordial as some *Archae* bacteria have both ecto-and-endo-symbiotic associations with protozoa (Finly et al., 1994). It has been demonstrated that saponins from different sources are toxic to protozoa and have been identified as possible defaunating agents in the rumen (Newbold et al., 1997). Their sensitivity towards saponins is due to the presence of sterols in protozoa, but not in bacterial, membranes (Williams and Coleman, 1992). Thus, the sterol-binding capacity of saponins most probably causes the destruction of protozoa cell membranes. Consequently, methanogens attached to protozoa usually decrease when protozoa numbers decline. It was noted in this study that the protozoa counts were reduced by 48.78, 49.18 and 52.84% and 45.52, 34.96, 72.35 and 67.1% at levels of 2, 4, 6 and 8 mg of tea and 48, 54, 60 and 66 mg for fenugreek, respectively (Table 1). Therefore, it is possible that the decrease in methane concentration in this study can be attributed to a decrease in numbers of protozoa and attached methanogens. Figure 1 show that methane concentration began to decline at 3h of incubation, reached the lowest level at 48h of incubation and then ascended gradually. This suggests that the antimethanogenic activity of saponins was transient. Probably, a new balance between hydrogen producers other than protozoa and free methanogens is established when ruminal fauna was reduced by saponins. This situation could also be explained by the adaptation of ruminal bacteria to the presence of saponins. It has been demonstrated that these moieties are degraded by ruminal microbiota and their deglycosylation attenuate their biological activity (Hart et al., 2008).

Ammonia in the rumen is produced from both dietary degradation and microbial lysis. In the present study, N-NH$_3$ concentrations were reduced by both PRS in a dose-dependent relationship. This reduction reached 49.47 and 72.02% for the highest levels of tea and fenugreek, respectively. The effect of saponins on rumen ammonia concentration is not consistent across studies. Busquet el al. (2006) reported that *Quillaja saponaria* and *Yucca schidigera* significantly reduced N-NH$_3$ concentration after 24h of incubation. In the same way, Hu et al. (2005 recorded that inclusion of tea to a mixed diet (grass and corn) in batch systems also decreased ammonia production. However, Muetzel et al. (2003) noted that saponin from *Sapindus rarak* did not inhibit feed protein degradation *in vitro*. Wand et al. (1998) found that the extract from *Yucca schidigera* enhanced casein degradation in continuous fermenters. According to Mao *et al*. (2010), the reduction in N-NH$_3$ concentration by saponins could be due either to decrease in urease activity or to capacity binding of saponins to ammonia. However, others researchs found that N-NH$_3$ concentration decrease is correlated to the decline in protozoa numbers (Hart et al., 2008; Goel et al., 2008; Wang et al., 2009). The observed decline in N-NH$_3$ concentration in this study is consistent with a reduction in protozoal counts.

The IVTDMD was significantly reduced with increasing levels of both PRS, which was similar to the observations made by Klita et al. (1996) and Hess et al. (2004). This observation was probably due to the inhibition of cellulolytic bacteria and fungal growth. Additionally, Rochfort et al. (2008) have reported that the effect of saponins was more pronounced against Gram positive bacteria like *Streptococcus bovis*.

Conclusion

The addition of PRS, tea and fenugreek, modified rumen fermentation and inhibited the release of methane and ammonia, which may be beneficial for improving nutrient utilization and animal growth. For future research, it will be interesting to see if these *in vitro* results could be

translated to *in vivo* activity and if the saponins can be administrated via plant feed or as plants extracts.

References

Agarwal, N., C. Shekhar, R. Kumar, L. C. Chaudhary and D. N. Kamra. 2009. Effect of peppermint (*Mentha piperita*) oil on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. Anim. Feed Sci. Technol. 148:321-327.

Blûmmel, M., H. Steingass and K. Becker. 1997. The relationship between *in vitro* gas production, *in vitro* microbial biomass yield and ^{15}N incorporation and its implications for the prediction of voluntary feed intake of roughages. Br. J. Nutr. 77:911-921.

Busquet, M., S. Calsamiglia, A. Ferret and C. Kamel. 2006. Plant extracts affect *in vitro* rumen microbial fermentation. J. Dairy Sci. 89:761-771.

Chaney, A.L. and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130-132.

Finaly, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley and R. R. Hirt. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117:157-162.

Francis, G., Z. Kerem, H. P. S. Makkar and K. Becker. 2002. The biological action of saponin in animal system: A review. Br. J. Nutr. 88:587-605.

Fuller, J. R. and D. E. Johnson. 1981. Monensin and lasalocid effects on fermentation *in vitro*. J. Anim. Sci. 53:1574-1580.

Goel, G., H. P. S. Makkar and K. Becker. 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.

Hart, K. J., D. R. Yãnez-Ruiz, S. M. Duval, N. R. McEwan and C. J. Newbold. 2008. Plant extracts to manipulate rumen fermentation. Anim. Feed Sci. Technol. 147:8-35.

Hess, H. D., M. Kreuzer, T. E. Díaz, C. E. Lascano, J. E. Carulla, C. R. Soliva and A. Machmüller. 2003. Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. Anim. Feed Sci. Technol. 109:79-94.

Hess, H. D., R. A. Beuret, M. Lotscher, I. K. Hindrichsen, A. Machmüller, J. E. Carulla, C. E. Lascano and M. Kreuzer. 2004. Ruminal fermentation, methanogenesis and nitrogen utilisation of sheep receiving tropical grass hay-concentrate diets offered with *Sapindus saponaria* fruits and *Cratylia argentea* foliage. Anim. Sci. 79:177-189.

Holtshausen, L., A. V. Shaves, K. A. S. Beauchemin, M. McGinn, T. A. McAllister, N. E. Odongo, P. R. Cheeke and C. Benchaar. 2009. Feeding saponin-containig *Yucca shidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. J. Dairy Sci. 92:2809-2821.

Hu, W. L., J. X. Liu, J. A. Ye, Y. M. Wu and Y. Q. Guo. 2005. Effect of tea saponin on rumen fermentation *in vitro*. Anim. Feed Sci. Technol. 120:333-339.

Jouany, J. P. 1982. Volatile fatty acids and alcohols determination in digestive content, silage juice bacterial culture and anaerobic fermentation content. Sci. Alim. 8:293-307.

Klita, P. T., G. W. Mathison, T. W. Fenton and R. T. Hardin. 1996. Effects of alfalfa saponins on digestive function in sheep. J. Anim. Sci. 74:1144-1156.

Lassey, K. R. 2007. Livestock methane emission from the individual grazing animal through national inventories to the global methane cycle. Agric. Forest Meteorol. 142:120-132.

Lovett, D. K., L. J. Stack, S. Lovell, J. Callan, B. Flynn, M. Hawkins and F.P. O'Mara. 2005. Manipulating enteric methane emissions and animal performance of late-lactation dairy cows through concentrate supplementation at pasture. J. Dairy Sci. 88:2836-2842.

Makkar, H. P. S. 2005. *In vitro* gas methods for evaluation of feeds containing phytochemicals. Anim. Feed. Sci. Technol. 123:291-302.

Makkar, H. P. S., G. Francis and K. Becker. 2007. Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. Anim. 1:1371-1391.

Mao, H. L., J. K. Wang, Y. Y. Zhou and X. J. Liu. 2010. Effects of tea saponins and soybean oil

on methane production, fermentation and microbial population in the rumen of growing lambs. Livest. Sci. 129:56-62.

Menke, K. H. and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Dev. 28:47-55.

Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz and W. Schneider. 1979. The estimation of digestibility and metabolizable energy content of ruminant feedstuffs from gas production when they are incubated with rumen liquor. J. Agric. Sci. 93:217-222.

Moss, A.R., J.P. Jouany and J. Newbold. 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49:231-253.

Muetzel, S., E. M. Hoffmann and K. Becker. 2003. Supplementation of barley straw with *Sesbania pachycarpa* leaves in vitro: effects on fermentation variables and rumen microbial population structure quantified by ribosomal RNA-targeted probes. Br. J. Nutr. 89:445-453.

Mwenya, B., B. Santoso, C. Sar, Y. Gamo, T. Kobayashi, I. Arai and J. Takahashi. 2004. Effects of including β-1–4-galactooligosaccharides, lactic acid bacteria or yeast culture on methanogenesis as well as energy and nitrogen metabolism in sheep. Anim. Feed Sci. Technol. 115:313-326.

Newbold, C. J., S. M. Elhassan, J. Wang, M. E. Ortega and R. J. Wallace. 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Br. J. Nutr. 78:237-249.

Ogimoto, K. and S. Imai. 1981. Atlas of rumen microbiology. Japan Scientific Society Press, Tokyo, Japan.

Orskov, E. R. and I. Mc Donald. 1979. The estimation of protein degradability in the rumen from incubation measurement weighted according to rate passage. J. Agric. Sci. 92:499-503.

Patra, A. K., D. N. Kamra and N. Agarwal. 2006. Effect of plant extracts on *in vitro* methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Anim. Feed Sci. Technol. 128:276-291.

Pen, B., C. Sar, B. Mwenya, M. Kuwaki, R. Morikawa and J. Takahashi. 2006. Effects of *Yucca schidigera* and *Quillaja saponaria* extracts on in vitro ruminal fermentation and methane emission. Anim. Feed Sci. Technol. 129:175-186.

Ramírez-Restrepo, C. A., T. N. Barry, A. Marriner, N. López-Villalobos, E. L. McWilliam, K. R. Lassey and H. Clark. 2010. Effects of grazing willow fodder blocks upon methane production and blood composition in young sheep. Anim. Feed Sci. Technol. 155:33-43.

Rochfort, S., A. J. Parker and F. R. Dunshea. 2008. Plant bioactves for ruminant health and productivity. Phytochem. 69:299-322.

Soliva, C. R., A. B. Zeleke, C. Clement, H. D. Hess, V. Fievez and M. Kreuzer. 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.

Statistical Analysis System Institute Inc. 1990. SAS/STAT® user's guide Int Vol 1, version 6, Fourth Edition, Cary, NC, USA.

Takahashi, J., B. Mwenya, B. Santoso, C. Sar, K. Umetsu, T. Kishimoto, K. Nishizaki, K. Kimura and O. Hamamoto. 2005. Mitigation of methane emission and energy recycling in animal agricultural systems. Asian Austral. J. Anim. Sci. 18:1199-1208.

Ungerfeld, E. M., S. R. Rust, R. J. Burnett, M. T. Yokoyama and J. K. Wang. 2005. Effects of two lipids on *in vitro* ruminal methane production. Anim. Feed Sci. Technol. 119:179-185.

Wang, C. J., S. P. Wang and H. Zhou. 2009. Influences of flavomycin, rapodiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Anim. Feed Sci. Technol. 148:157-166.

Wang, Y., T. A. McAllister, C. J. Newbold, L. M. Rode, P. R. Cheeke, K. J. Cheng. 1998. Effect of *Yucca schidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). Anim. Feed Sci. Technol. 74:143-153.

Williams, A. G. and G. S. Coleman. 1992. The rumen protozoa. Springer-Verlag Inc., New York, USA. pp. 441.