Indian Journal of Animal Sciences **83** (8): 802–805, August 2013/Article

Effect of graded level Egyptian clover (*Trifolium alexandrium)* supplementation on *in vitro* methane production and fermentation pattern

P K MALIK[1], K K SINGHAL[2], N M SOREN[3], A THULASI[4], L JOSE[5] and S S SANTOSH[6]

National Dairy Research Institute, Karnal, Haryana 132 001 India

Received: 7 September 2012; Accepted: 12 January 2013

ABSTRACT

Saponin content of Egyptian clover at 3 different cuts was determined and second cut fodder was selected for the supplementation @ 0 (T_0), 15 (T_1), 30 (T_2) and 45 (T_3)% to wheat straw based diet to study the effect on *in vitro* methane production and fermentation pattern. Saponin level of the diet after inclusion of fodder was 0, 0.15, 0.30 and 0.45%, respectively in T_0, T_1, T_2 and T_3. Total gas production was significantly higher in T_3 and T_2 as compared to control group. On the inclusion of clover, a reduction of 9.6, 15.2 and 23.6% in methane production was noticed in T_1, T_2 and T_3, respectively. Decreasing proportion of methane in total gas with increasing levels of supplementation reflected that saponin up to 0.45% of diet did not affect the fermentability adversely. Maximum reduction in protozoal numbers was observed in treatment T_3. Significant increase in DM degradability as compared to control was reported in treatment T_2 and T_3. Likewise TVFA production (mmol/L) also increased considerably on berseem fodder supplementation. Looking at the efficacy of saponin containing Egyptian clover, it may be inferred that there is an urgent need to explore the newer feed resources possessing substantial saponin and to establish their safe inclusion level in diet of ruminants.

Key words: Berseem fodder, Egyptian clover, Fermentation, *In vitro* methane, Saponin

The current atmospheric methane concentration is 1,782ppb, almost 155% higher than the pre-industrial concentration (IPCC 2007, Rawat *et al*. 2007). Livestock sector alone is dispensing huge quantity of methane (75–80 Tg/y) in the atmosphere. India being the major livestock holder in the world contributes substantially to methane emission and the situation is further worsened by the type of feeds and fodders available for the livestock. Various plant biomolecules including saponins are reported to possess anti-methanogenic action either through direct or indirect way. Saponin content of alfalfa, *Sesbania sesban*, *Quillaja saponaria, Acacia auriculoformis* and *Yucca schidigera* is well documented but only one report on the saponin content of Egyptian clover (*Trifolium alexandrium*) is available (Fernanded and Waditake 2006). Therefore an attempt was made to determine the saponin content of Egyptian clover and study the effect of its graded supplementation on *in vitro* methane production and fermentation pattern.

MATERIALS AND METHODS

Egyptian clover (*Trifolium alexandrium*) collected from the farm at first (60 DAS i.e. days after sowing), second (90 DAS) and third cut (120DAS) were brought to the laboratory for DM estimation at 80±2°C. Oven dried samples were ground and used for the saponin determination following the methodology of Yosioka *et al.* (1974) using methyl and n-butyl alcohol. Egyptian clover of second cut due to highest saponin content was selected for the graded supplementation @ 0(T_0), 15(T_1), 30(T_2) and 45(T_3)% to wheat straw based diet. Roughage to concentrate ratio was kept as 60: 40 with 12–13% CP by changing the proportion of concentrate ingredients. Rumen liquor for the *in vitro* study was collected from the 3 crossbred male rumen fistulated cattle (BW 284.33 kg) fed on wheat straw and concentrate based diet and filtered through 4 layers of muslin cloth. 0.2 g substrate was incubated in 100 ml well lubricated glass syringe with 20 ml Menke's buffer and 10 ml strained rumen liquor. The piston of the syringe was adjusted to the surface of liquid to ensure the removal of air in the syringe and outlet was connected with a silicon rubber tube of 4–5 cm length through 3-way valve by a plastic clip. For determining the proportion of methane in gas sample, gas chromatograph fitted with flame ionization detector (FID) and Porapak 'N' column was used. Simultaneously, kinetics of total gas and methane with coefficients of determination (R^2) was also derived by using the Sigma stat (2001) to fit the data in exponential model of

Present address: [1,3-6]Senior Scientist (malikndri@gmail.com), NIANP, Bengluru. [2]Principal Scientist and Retired Head (k_ksinghal@yahoo.co.in).

Ørskov and Mc Donald (1979). At the end of incubation period (96 h), *in vitro* dry matter degradability (IVDMD) was determined. Simultaneously, total volatile fatty acid (TVFA) and individual fatty acids were estimated as per Barnett and Reid (1957) and Erwin *et al.* (1961), respectively. However, protozoal enumeration was done by staining (Santra *et al.* 1998). The data thus obtained on gas production, fermentation pattern and protozoal counts were analyzed using ANOVA as per the procedure of Snedecor and Cochran (1986).

RESULTS AND DISCUSSION

Saponin content: The average saponin content (% DM basis) of Egyptian clover irrespective of cut was recorded as 0.83% however, among all the 3 cuts, the highest ($P<0.05$) saponin content was recorded in second cut and lowest in first cut (Table 1). The saponin level in first and third cut was at par and not substantially differs. Sapnonin content of berseem fodder at different cuts in the present study was within the range of saponin reported by Fernandes and Waditake (2006) in 16 cultivars of berseem. They reported saponin content in 16 different cultivars in the range of 0.26 to 1.10%. The variation in saponin levels of different cultivars may be attributed to the genetic factors of cultivars (Fernandes and Waditake 2006) or legume species as well as agronomic practices (Livingston *et al*. 1979). Our results revealed that saponin in fodder was lowest at the initial stage of growth and increased with the plant maturity up to the flowering stage and thereafter, declined almost up to the level of first cut. Similar to the observations, due to 36% higher saponin content in second cut of Egyptian clover, it was taken further for *in vitro* study to ascertain the effect on fermentability and methane production.

Effect on total gas: Highest total gas production (l/kg DM) was recorded in T_3 treatment supplemented with saponin containing Egyptian clover @ 45% of the diet with a total saponin of 0.45% of the diet (Table 1). Total gas production in T_3 was significantly ($P<0.05$) higher than that recorded in control (T_0). The supplementation of Egyptian clover at 45% level lead about 24% more gas production as compared to control group. Similarly, gas production in T_2 treatment was also higher ($P<0.05$) than the gas production in control, however, total gas production in T_1 treatment on the supplementation of berseem fodder at 15% level did not differ significantly from the control group. Likewise the gas production between T_1 and T_2 and T_2 and T_3 did not vary significantly (Table 1). The increase in total gas production at 30 and 45% levels to the wheat straw based diet revealed that the saponin content from Egyptian clover (second cut) up to 0.45% of the diet did not affect the fermentability of diet and subsequent gas production. Higher ($P<0.05$) gas production in different treatments due to legume supplementation may be attributed to consequent associative effect, which increases the fermentability of wheat straw based diet by colonizing the fibre for microbial action. Comparatively higher fermentation (almost 21%) of the slowly but fermentable fraction was recorded in T_3 than the control group at 45% level (Table 1). The rate of gas production (l/h) expressed by c fraction was highest on 30% supplementation (0.164 l/h) and lowest in the treatment without berseem fodder (T_0). Higher proportion of legumes fastens the rate of fermentation (c) with lower asymptotic gas production, owing to the surface properties of the cell wall of dicotyledons (Lopez *et aI.* 1991). Lower value of legumes might be due to higher lignin and CP content (Menke and Steingass 1988). Data from the study suggested that the supplementation of Egyptian clover at 45% level did not increase fermentability of diet than that recorded at 30% levels. Higher gas production on the supplementation of legumes was reported by others also (Monforte-Briceno *et al.* 2005).

Table 1. Effect of berseem fodder supplementation on cumulative totals gas, *in vitro* methane production and protozoal counts

Parameter	Treatment			
	T_0	T_1	T_2	T_3
Total gas (l/kg DM)	106.90^a	115.80^{ab}	125.55^b	132.65^{bc}
b	118.8	127.5	136.05	144.75
c	0.137	0.154	0.164	0.161
R^2	0.98	0.97	0.96	0.96
Methane (l/kg DM)	21.29^c	19.24^{bc}	18.05^{ab}	16.25^a
b	23.56	20.83	19.63	17.40
c	0.0302	0.0336	0.0335	0.0353
Methane as% of TG	19.92	16.61	14.38	12.25
Protozoal counts (log cfu/ml)	4.74^c	3.69^{bc}	3.44^b	2.84^a

Effect on in vitro *methane production and protozoa:* Data given in Table 1 on *in vitro* methane production revealed highest methane production in control group (T_0), which reduced drastically on the graded supplementation on Egyptian clover to wheat straw based diet. The reduction in methane production as compared to control was 9.6, 15.2 and 23.6% in the treatment T_1, T_2 and T_3, respectively. The highest and significant ($P<0.05$) reduction in methane production in T_3 in comparison to T_0 and T_1 may be attributed to its saponin content, highest among the 4 treatments (0.45 v/s 0.3 and 0.15%). Likewise methane production in the treatment T_2 also reduced significantly than the production recorded under T_0 treatment. However, the variation in methane production between T_0 and T_1; T_1 and T_2 and T_2 and T_3 was comparable ($P>0.05$). Decrease in methane production on the supplementation of Egyptian clover (second cut) may be due to a reduction in protozoal numbers (Table 1), which is accountable for making the substrate hydrogen available to the Archaea who use this for the reduction of CO_2 to methane. Reduction in methane on the feeding of forage legumes was reported by others also (Reddy and Krishna 2009). Recently Feng *et al.* (2012) also reported significant decrease in methane production on the addition of gross saponins from the fodder *Tribulus terrestris* found in China. Thus, decrease in methane production on supplementation of berseem fodder particularly at 30 and 45% levels was due to change in fermentation pattern towards more propionate production at the cost of acetate as evidenced from the results of this study (Table 2). As far as kinetics of methane production is concerned, lowest methane production from slowly fermentable fraction "b" was recorded in T_3 (17.40) and highest in T_1. For the rest 2 treatments, methane production lies in the range of these 2 treatments only (Table 1). With increasing fermentability and consequently total gas production on the saponin rich clover supplementation, the proportion of methane in total gas decreased accordingly reflected that reduction in methane was achieved without compromising fermentability and saponin at the level of 0.45% of the total diet did not show any adverse effect. The decrease in methane production from the slowly fermentable fraction was 11, 16 and 26% respectively in T_1, T_2 and T_3. Reduction in protozoal numbers (log cfu/ml) was noticed on the supplementation on berseem fodder at the graded level and maximum reduction was seen in treatment T_3 having highest saponin content. The detergent action of saponin is believed to be responsible for killing the rumen protozoa (Makkar *et al.* 1998). Results of the study are in consonance of the findings of Klita *et al.* (1996), who reported a significant reduction in ruminal protozoal numbers on alfalfa root saponins supplementation in sheep diet.

Fermentation pattern: Supplementation of Egyptian clover (second cut) to wheat straw based diet increased *in vitro* dry matter digestibility in the tune of 7–22% among different treatment groups. The significant increase in the DM degradability was reported in treatment T_2 and T_3 as compared to control group (Table 2). However, the digestibility in T_2 and T_3 treatment was higher than that recorded in T_1 but the variation was not significant. The increase in *in vitro* DM degradability on graded supplementation may be attributed to the high soluble carbohydrates and better fibre quality of nutritious Egyptian clover than the wheat straw. Moreover, total volatile fatty acid (mmol/L) production also increased on the supplementation of Egyptian clover with highest and lowest production rate in T_3 and T_0, respectively (Table 2). The increase in TVFA production was due to increased fermentability of diet on supplementation. As the proportion of berseem fodder in wheat straw and concentrate based diet increased, a shift in the fermentation pattern was recorded towards propionate; at the cost of acetate (Table 2). The results from study are in consonance of the findings from many studies (Reddy *et al.* 1991, Abreu *et al.* 2004).

On the basis of the results, it may be concluded that the supplementation of Egyptian clover (second cut) at 45% of diet decreased methane production significantly without affecting digestibility and fermentability of diet. Therefore, saponin up to a level of 0.45% of the diet from natural feed sources may be included in diet. Looking at the urgency of methane mitigation from Indian livestock, priority should be given to identify newer saponin rich feed stuffs and establishing their safe level of inclusion in the ration for achieving the goal.

Table 2. DM degradability and VFA production (mmol/L) as affected by graded supplementation of berseem fodder

Parameter	Treatment			
	T_0	T_1	T_2	T_3
IVDMD (%)	43.72^a	46.89^{ab}	49.70^b	53.41^b
TVFA (mmol/L)	101.43^a	102.42^a	107.88^b	108.37^b
Acetate (%)	74.66^b	73.34^{ab}	70.09^a	69.56^a
Propionate (%)	16.34^a	17.55^a	21.09^b	21.07^b
Butyrate (%)	9.0	9.11	8.82	9.37
A: P	4.5: 1	4.1: 1	3.3: 1	3.3: 1

REFERENCES

Abreu A, Carulla J E, Lascano C E**,** Díaz T E, Kreuzer M and Hess H D. 2004. Effects of *Sapindus saponaria* fruits on ruminal fermentation and duodenal nitrogen flow of sheep fed a tropical grass diet with and without legume. *Journal of Animal Science* **82**: 1392–1400.

Barnett A, John G and Reid R L. 1957. Studies on the production of volatile fatty acids from grass by rumen liquor in an artificial rumen. 1. The volatile acid production from fresh grass. *Journal of Agricultural Science* **48**: 315–21.

Erwin E S, Marco G J and Emery I N. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *Journal of Dairy Science* **44**: 1768–71.

Feng Z H, Feng Yu, Yan Z G, Qiu F L and Jian G L. 2012. Effect of gross saponin of *Tribulus terrestris* on ruminal fermentation and methane production *in vitro*. *Journal of Animal and Veterinary Advances* **11**(12): 2121–25.

Fernandes A P and Waditake S K. 2006. Comparative evealuation of berseem (*Trifolium alexandrinum*) verities for yield and fodder quality. *Animal Nutrition and Feed Technology* **6**(2): 301–06.

IPCC. 2007. *4th Assessment Report of the United Nations*. Intergovernmental Panel on Climate Change.

Klita P T, Mathison G W, Fenton T W and Hardin R T. 1996. Effects of alfalfa root saponins on digestive function in sheep. *Journal of Animal Science* **74**: 1144–56.

Livingston A L, Knuckles B E, Edwards R H, de Fremery D, Miller R E and Kohler G O. 1979. Distribution of saponin in alfalfa protein recovery systems. *Journal of Agricultural and Food Chemistry* **27**: 362–65.

Lopez S, Carro M D, Gonzalez J S and Ovejero F J. 1991. Rumen degradation of the main forage species harvested from permanent mountain meadows in North-western Spain. *Journal of Agricultural Science* **117**(3): 363 – 69.

Makkar H P S, Sen S, Blümmel M and Becker K. 1998. Effects of fractions containing saponins from *Yucca shidigera*, *Quillaja* *saponaria*, and *Acacia auriculoformis* on rumen fermantation. *Journal of Agricultural and Food Chemistry* **46**: 4324–28.

Menke K H and Steingass H. 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* **28**: 7–55.

Monforte-Briceño G E, Sandoval-Castro CA, Ramírez-Avilés L and Capetillo-Leal C M. 2005. Defaunating capacity of tropical forage trees. Effect of PEG and its relationship with *in vitro* gas production. *Animal Feed Science and Technology* 123–124: 313–27.

Rawat M, Singh U K, Mishra A K and Subramanian V. 2007. Methane emission and heavy metals quantification from selected landfill areas in India. *Monitoring and Assessment* **137**: 67–74

Reddy D V and Krishna N. 2009. Precision animal nutrition: A tool for economic and eco-friendly animal production in ruminants. *Livestock Research for Rural Development* **21**(3): 36.

Reddy D V, Singh V B and Mehra U R. 1991. Effect of feeding green berseem in improving rumen degradation kinetics of straw. *Indian Journal of Animal Sciences* **61**(2): 199–201.

Santra A, Karim S A, Mishra A S, Chaturvedi O H and Prasad R. 1998. Rumen ciliate protozoa and fiber digestion in sheep and goats. *Small Ruminant Research* **30**: 13–18.

Snedecor G W and Cochran W G. 1986. *Statistical Methods.* Oxford and IBH Publishing Co., New Delhi.

Yosioka *et al.* 1974. Estimation of saponin. *Tetrahedron* **30**: 707–14.