EFFECT OF ALFALFA FODDER SUPPLEMENTATION ON ENTERIC METHANE EMISSION MEASURED BY SULFUR HEXAFLUORIDE TECHNIQUE IN MURRAH BUFFALOES

P.K. Malik[1,*] and K.K. Singhal[2]

ABSTRACT

Phyto-sources possessing different secondary metabolites are under investigation for the mitigation of enteric methane emission from livestock. Alfalfa (*Medicago sativa*) contains considerable saponin which is known for methane reduction through anti-protozoal action. Therefore, this study was undertaken to ascertain the effect of saponin containing alfalfa fodder (*Medicago sativa;* second cut) on enteric methane emission in Murrah buffaloes quantified using sulfur hexafluoride (SF_6) technique. Twelve male Murrah buffalo calves were randomly divided into two groups of six animals each. Buffalo calves in control group were fed on wheat straw and concentrate based diet (R: C, 60:40), while animals in test group were supplemented with saponin containing alfalfa fodder (second cut, 30%) replacing wheat straw on w/w basis. Effect of alfalfa fodder supplementation on rumen fermentation characteristics, archaeal and protozoal population were also studied. Enteric methane emission in control and test group buffaloes was reported as 78.09 and 61.39 g/d, respectively. In this study, about 21% reduction in enteric methane emission was achieved on the feeding of saponin containing alfalfa fodder at 30% level of the diet. However, dry matter intake, pH, ammonia nitrogen and total volatile fatty acid production did not differ ($p>0.05$) among the groups. A significant ($p<0.05$) decrease in acetate production was also with concomitant increase in propionate production. Results revealed a non-significant change in archaeal population, while protozoal population were adversely affected and about 20% less numbers were observed in test group. From the study it may be concluded that saponin from natural feed resources like alfalfa fodder at a level of 6.0 g/kg DM can be used for the significant enteric methane reduction.

Keywords: enteric methane, leguminous fodder, Murrah buffaloes, saponin, sulfur hexfluoride

INTRODUCTION

Current atmospheric methane concentration is 155% higher than the pre-industrial concentration (IPCC 2007). Livestock are the major contributor to anthropogenic methane wherein Indian livestock contributes substantially to global enteric methane emission. Due to the huge dispension of methane, United Nation's Food and Agriculture Organization recently stated livestock are the major threat for environment. As per one

[1]Animal Nutrition, ICAR-National Institute of Animal Nutrition and Physiology, Bangalore, India, *E-mail: malikndri@gmail.com

[2]National Dairy Research Institute, Karnal, India

estimate, approximately 37% of anthropogenic methane and 12-13% of the total atmospheric methane is emitted from livestock. Asia is the harbor for 179.5 million buffaloes and India alone is possessing approximately 56.7 percent of the total (FAO, 2008). During last 10 years, the world buffalo population increased at a rate of 1.49% per year, wherein, in India and Asia, the increase was 1.53% and 1.45% per annum, respectively. Various agencies have promulgated the enteric methane emission from Indian livestock in the tune of 7.2-12.9 Tg/y (Malik *et al.,* 2012). Buffaloes are the noteworthy emitters to this and contribute about 2.8 Tg/y (Singh, 1998). The need for curtailing the methane emission from ruminants is mandatory from global warming and dietary energy loss point of view (Malik *et al.*, 2013).

Worldwide attempts have been made for the mitigation of livestock methane using halogenated methane analogues, antibiotics, fat and oils, organic acids, but the response from majority of these was highly variable and adoption rate due to high cost, reduced feed intake, toxicity to inhabiting rumen microbes/host animal and transitory effect was very low (Malik *et al.,* 2012). Plant secondary metabolites such as saponin may be a potent agent in achieving the significant reduction in methane emission from livestock. So far *in vitro* studies have been conducted to evaluate the effect of saponin on methane production (Malik and Singhal*,* 2008; Wang *et al.,* 2011), and reports for methane reduction in buffaloes on using saponin from natural sources is very limited. Keeping these facts in view, the study was under taken to assess the effect of saponin containing alfalfa fodder supplementation on enteric methane emission in buffaloes.

MATERIALS AND METHODS

Animals and feeding

Twelve male Murrah buffalo calves (BW 172.62±0.36 Kg) were selected from the herd of institute and divided into two groups of six animals each. Necessary permission from the Animal Ethical Committee was obtained for conducting the experiment. Buffalo calves were kept in a well ventilated shed having a provision for individual feeding. To keep the animals free from external and internal parasites, butox 0.5% (v/v) and albandazole (0.5 mg/kg BW) were given, respectively. Buffalo calves were fed as per the Kearl (1982) to meet the nutritional requirement. Animals in group I (control) was fed on wheat straw and concentrate based ration (60:40). Concentrate mixture comprising maize grain (33%), groundnut cake (20.2%), mustard cake (12%), wheat bran (20%), deoiled rice bran (11%), urea (0.8%), mineral mixture (2%) and common salt (1%) was offered to the animals of control group. Animals under group II received the total mixed ration consisting wheat straw, concentrate and second cut alfalfa fodder (*Medicago sativa*) in the ratio of 30:40:40. Concentrate mixture for the group II animals was prepared by mixing of maize grain (24%), groundnut cake (15%), mustard cake (8%), wheat bran (42%), deoiled rice bran (8%), mineral mixture (2%) and common salt (1%). Second cut alfalfa (*Medicago sativa*) fodder was selected for the supplementation to buffalo calves on the basis of comparatively higher saponin content among first three cuts. The saponin content of alfalfa fodder was determined as per the method of Yosioka *et al.* (1974). The CP was maintained about 12.0% of the diet for both control and test group.

Measuring methane emission using Sulfur Hexafluoride (SF_6) tracer technique

In vivo methane emission from Murrah buffalo calves fed on control and test diet was measured using sulfur hexafluoride (SF_6) trace technique of Johnson *et al*. (1994). Four successful methane gas collections from each individual buffalo were made to quantify the daily emission under control and test group. Before actual gas collection from the calves, PVC canisters were fixed around the neck of each animal for 2 days to acclimatize them. The gas was collected into PVC canister through halters tied around the head and in front of nostrils. The initial and final pressure of the PVC canisters weres measured at the time of tying and removal. Brass Permeation tubes (brass, 1.25" length, 3x 16" ID) with central window were filled with SF_6 gas fitted with Teflon cap and 2µ brass frit using standard protocol. Frit was fixed to facilitate the release of SF_6 from brass permeation tube kept at room temperature. Daily weight of permeation tube was recorded until the release rate of SF_6 became constant, thereafter; the permeation tubes were placed into rumen. The SF_6 and CH_4 emitted from each animal were collected into an evacuated yoke like PVC canister through a capillary tube ending just above the nostrils of the animal (Figure 1). A similar canister was hanged in animal shed to record background concentration of CH_4. Concentration of CH_4 and SF_6 gases in collected gas samples was determined using gas chromatograph fitted with Flame Ionization Detector (FID) and Electron Capture Detector (ECD) for methane and SF_6, respectively. The methane emission rate was determined from CH_4 to SF_6 ratio using release rate of SF_6 as given in formula. Background methane was subtracted from methane concentration in the PVC canister.

$$QCH4 = \frac{QSF_6 \times [\{CH_4\}Y - \{CH_4\}b]}{[SF_6]}$$

QCH_4 is methane emission rate; QSF_6 is the release rate of SF6; CH_4b is the concentration of methane in background sample and CH_4 Y is the methane concentration in PVC canister.

Rumen fermentation, archaeal and protozoal population

At the end of gas collection, rumen liquor samples were collected from buffalo calves for three consecutive days through stomach tube (Figure 2). Rumen liquor was stained through four layers of muslin cloth and stored into an insulated anaerobic container. Rumen liquor pH was recorded using digital pH meter just after the collection of liquor. However, ammonical nitrogen, total volatile fatty acids (TVFA) and volatile fatty acids (VFA) fractionation were determined using standard protocol of Conway (1950), Barnett and Reid (1957) and Erwin *et al*. (1961), respectively. Methanogenic archaea as well as protozoal population were also enumerated in rumen liquor samples collected from buffalo calves fed on control and test diet. Anaerobic media described by Ranade and Gadre (1988) for methanogens was used for enumerating the numbers while protozoal numbers was enumerated through haemocytometry microscopic counting method. Data were analyzed in SPSS 16 using one way Anova and means were compared for statistical difference.

RESULTS AND DISCUSSION

***In vivo* methane emission**

Saponin content (DM basis) of alfalfa

Figure 1. *in vivo enteric* methane emission measurement in Murrah buffalo calves using SF_6 technique.

Figure 2. Rumen liquor collection from buffaloes using stomach tube.

fodder at three different cuts varied between 0.92±0.05 to 2.0±0.05 per cent with highest level at second cut (2.0%). Due to higher saponin level and results from *in vitro* studies (data not presented), second cut alfalfa fodder was selected for the supplementation at 30% level in wheat straw and concentrate based diet to ascertain the effect on enteric methane emission in Murrah buffalo calves. Similar to the findings of this study, Cheeke and Shull (1985) also reported highest saponin level in alfalfa fodder at second cut.

Dry matter intake (DMI) and *in vivo* methane emission as affected by the supplementation of saponin containing alfalfa fodder in Murrah buffaloes is depicted in Table 1 and Figure 3, respectively. Data did not reveal any significant change in dry matter intake between control and test group (Table 1). *In vivo* enteric methane emission in buffaloes in control and test groups is presented in Figure 3. Enteric methane emission in control and test group was 78.09 and 61.39 g/d, respectively. Enteric methane emission (g/d) in control group was significantly higher ($P<0.05$) than the test group. About 21% reduction in methane emission was reported in test group on the inclusion of saponin containing alfalfa fodder at 30% level replacing wheat straw in the diet (Figure 3). Similarly, *in vivo* methane emission on g/kg DMI was also significantly ($P<0.05$) lower in test group than control (18.1 vs. 12.4 g). Enteric methane emission on g/kg digestible dry matter intake basis was also significantly ($p<0.05$) lower in test group. Lower enteric methane emission from the test group buffaloes may be attributed to the saponin of alfalfa fodder. Average saponin intake in test group was 47 g/d or 6.03 g/kg of dry matter. Srivastava and Garg (2002) reported 19.26 g methane emission per kg of DMI in crossbred calves fed on paddy straw and fodder based diet. Mohini and Singh (2001) recorded the

Table 1. Effect of saponin containing alfalfa fodder supplementation on dry matter intake (DMI) and fermentation pattern in buffaloes.

Parameter	Control diet	Test diet
DMI		
Kg/d	7.40±0.28	7.79±0.19
Kg/100kg BW	2.77±0.16	2.94±0.08
g/kg$W^{0.75}$ BW	111.8±0.46	118.6±0.40
Fermentation		
pH	6.40±0.18	6.30±0.18
NH_3-N (mg/100ml)	30.92±1.37	28.62±1.33
TVFA (mM/l)	108.94±0.68	108.87±0.97
Acetate	74.13^a±0.34	67.07^b±0.35
Propionate	15.99^b±0.74	17.70^a±0.37
Butyrate	9.86±0.42	10.22±0.64
A:P	4.6:1	3.7:1

Values bearing a, b superscripts in a row differ significantly ($p<0.05$).

methane emission in the range of 15.97 - 18.35 g/kg DMI from buffalo calves fed on maize fodder and straw based diet. Mao *et al.* (2010) also found a reduction of 27.2% in methane emission from lamb on the inclusion of saponin at 4.1 g/kg level from *Camellia scinensis*. However, administration of 5 g/kg of *S. saponaria* fruits to sheep for 21 days reduced CH_4 emission by 7.8% only in a study of Hess *et al.* (2004).

Data from the study revealed that enteric methane emission in buffaloes is influenced by the saponin from alfalfa fodder. Lower methane emission in test group was attributed to the anti-protozoal action of saponin of alfalfa, which in turn puts a restriction on hydrogen transfer to methanogens (Krumholz *et al*., 1983). Mechanistically, saponin forms an irreversible complex with cholesterol which is an integral component of protozoal cell membrane and therefore leads to cell lysis and death.

Effect on feed fermentability

Dry matter intake (DMI) and fermentation characteristics as affected by the supplementation of saponin containing alfalfa fodder in Murrah buffaloes are presented in Table 1. Dry matter intake (DMI) did not show any significant change due to the supplementation (Table 1). Similarly, pH, ammonia nitrogen and total volatile fatty acid also did not affect with saponin containing alfalfa fodder supplementation. Data from the study envisaged that saponin from alfalfa fodder at 6.03 g/kg DM (total intake 47g) did not have any adverse effect on dry matter intake, and total volatile fatty acid production.

Acetate production ($P<0.05$) in alfalfa fodder supplemented buffaloes decreased significantly ($p<0.05$) by 7 units (9.5%) as compared to control (Table 1). On the other hand, propionate fraction at the cost of acetate significantly ($P<0.05$) increased, while butyrate remain unaffected by the supplementation. Data showed a shift in individual fatty acid production from acetate to propionate on the inclusion of alfalfa fodder. These results are in consonance of the findings of Diaz *et al.* (1993); Hristov *et al.* (1999) who also reported similar trend on the dietary incorporation of *Sapindus saponaria* and *Yucca schdigera* , respectively. Hu *et al.* (2006) also recorded same trend without affecting TVFA concentration on the incorporation of *Yucca* and *Quillaja saponin* in corn meal and grass based diet. From these results, it may be concluded that the saponin from alfalfa fodder to a level of 47 g/d or 6 g/kg DM did not affect the feed fermentability or dry matter intake.

Effect on methanogen archaea and protozoa

Results of the study revealed a non-significant effect of alfalfa fodder as such or its saponin (47g/d or 6.03g/kg DM) on rumen methanogen archaea. On the feeding of control as well as test diet, their population remained constant ~ 1.73 log CFU/ml (Figure 4), which shows that the saponin did not have any direct action on methanogen archaea and directly not accountable for methane reduction from buffalo calves. Results is in congruence of the findings of Wina *et al.* (2005) who did not find any decrease in methanogens number on the addition of saponin from *Sapindus rarak.* On the contrary the protozoal population was adversely affected due to alfalfa fodder supplementation (Figure 4). Protozoal population badly affected ($P<0.05$) in group II on the addition of saponin containing alfalfa fodder and the decrease was almost 20 per cent as compared to control group (Figure 4). Entodinimorphs are the most vulnerable to dietary changes in cattle and buffaloes (Bhatia *et al.*, 1998) and most susceptible

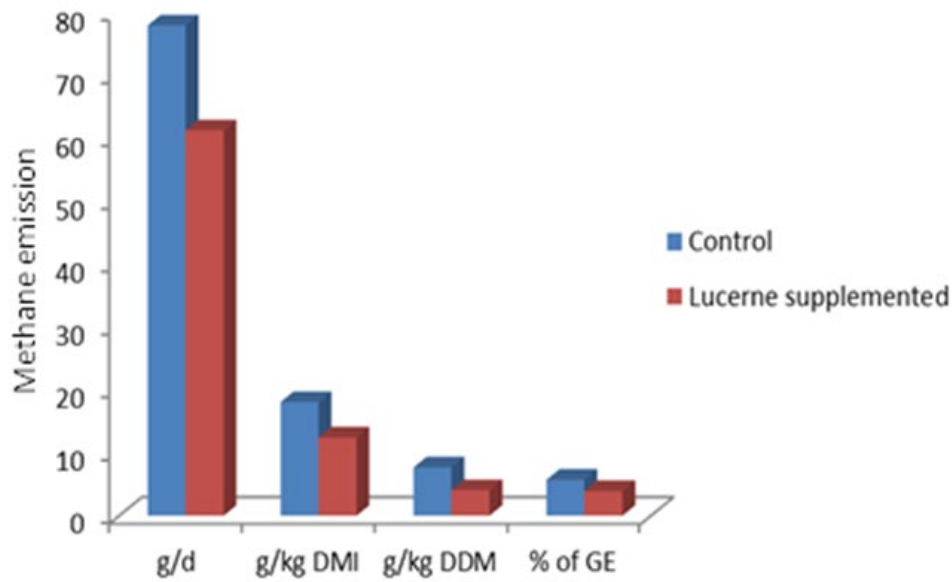

Figure 3. Effect of lucerne fodder supplementation on methane emission in Murrah buffaloes.

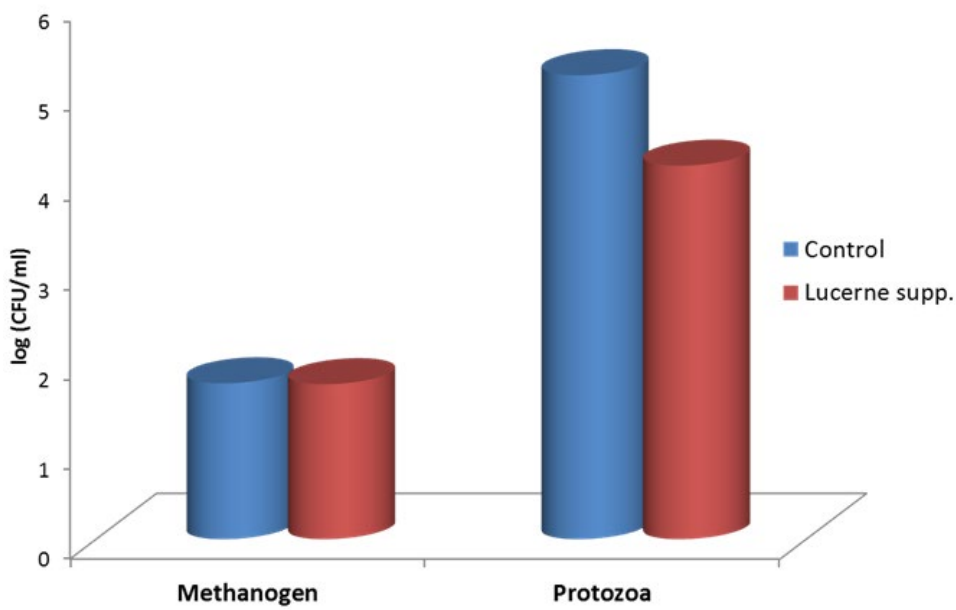

Figure 4. Effect of supplementation on rumen methanogens and protozoa.

to the dietary saponin (Lu and Jorgenson, 1987). However, in present study no attempt was made for the characterization of various protozoal population affected by alfalfa saponin. These results are in agreement with findings of Navas-Camacho *et al.* (1993); Diaz *et al.* (1993); Klita *et al*. (1996), noted a significant reduction in ruminal protozoa following dietary incorporation of *E. ciclocarpum, Sapindus saponaria* and alfalfa root saponin, respectively. Thus, the significant reduction in methane emission in group II on the inclusion of alfalfa fodder is attributed to comparatively lesser number of protozoa badly hit by saponin. Anti-protozoal effect of saponin is depend on the presence of cholesterol in protozoal cell membrane, which possible made a selective susceptibility of ruminal protozoa to saponin and due to this reason methanogens (lacking in cell membrane cholesterol) did not directly hit by saponin.

CONCLUSION

It may be inferred from the study that secondary metabolite saponins from natural feed sources like alfalfa may significantly reduce enteric methane emission in buffaloes and quite safe to feed up to a level of 6.03 g/Kg of DM or 47 g/day without affecting dry matter intake. Methanogenic archaea is not affected by the alfalfa fodder supplementation, while it has an adverse action on rumen protozoa which lead to less enteric methane emission. There is a need to conduct instant research towards exploring the saponin containing phyto-sources and to optimize their safe level of inclusion in diet for the substantial methane reduction.

REFERENCES

Barnett, A. and R.L. Reid. 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. *J. Agri. Sci.*, **48**: 315-21.

Bhatia, S.K., K. Pradhan, S. Singh and S. Singh. 1998. Microbes and their activities in rumen of cattle and buffaloes (*Technical bulletin*). Published by CCS Haryana Agricultural University, Hisar, India.

Cheeke, P.R. and L.R. Shull. 1985. *Natural Toxicants in Feeds and Poisonous Plants*. AVI Publishing Company, Inc., Westport, Conn., USA. 492p.

Conway, E.J. 1950. *Microdiffusion Analysis and Volumetric Error*, 4th ed. London: Crosby Lockwood. 391p.

Diaz, A., O.M. Avendon and A. Escobar. 1993. Evaluation of *Sapindus saponaria* as a defaunating agent and its effect on different ruminal digestion parameters. *Lives. Res. and Rural Dev.*, **5**: 1-10.

Erwin, E.S, G.J. Marco and I.N. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.*, **44**: 1768-1771.

FAO. 2008. The state of food and agriculture. Food and Agriculture Organisation, Rome. http:// apps.fao.org.page/collection. *Fao.org/stat 2008.*

Hess, H.D., M. Kreuzer, T.E. Diaz, T.E. Lascano, J.E. Carulla, C.R. Soliva and A. Machmuller. 2003. Saponin rich tropical fruit affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Anim. Feed Sci. and Tech.*, **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 utilization of sheep receiving tropical grass hay-concentrate diets offered with *Sapindus saponaria* fruits and *Cratylia argentea* foliage. *Anim Sci.*, **79**: 177-189.

Hristov, A.N., T.A. McAllister, F.H. Van Herk, K.J. Cheng, C.J. Newbold and P.R. Cheeke. 1999. Effect of *Yucca schidigera* on ruminal fermentation and nutrient digestion in heifers. *J. Anim. Sci.*, **77**: 2554-2563.

Hu, W.L, J.X. Liu, Yan-Qiu Guo, Yue-Min Wu. 2006. Effect of saponins on rumen fennentation and methanogenesis in vitro, p. 12l. *In Proceedings of 12th AAAP Congress*. Busan, South Korea (Abstract).

Inter Governmental Panel on Climate Change (IPCC). 2007. *Summary for Policymakers.* Approved in Plenary XXVII, working group contributions to the *IV assessment Report*, Valentia, Spain.

Johnson, K.A., M.T. Huyler, H.H. Westberg, B.K. Lamb and B. Zimmerman. 1994. Measurement of methane emission from ruminant livestock using SF6 tracer technique. *Environ. Sci. Tech.*, **28**: 359.

Kearl, L. 1982. *Nutrient requirements of ruminant in developing countries*. Utah State Univ. Logam, USA.

Kilta, P.T., G.W. Mathison and T.W. Fenton 1996. Effect of alfalfa root saponins on digestive function in sheep. *J. Anim. Sci.*, **74**: 1144-1156.

Krumholz, L.R., C.W. Forsberg and D.M. Veira. 1983. Association of methanogenic bacteria with rumen protozoa. *Can. J. Microbiology*, **29**: 676-680.

Lu, C.D. and N.A. Jorgensen. 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. *J. Nutr.*, **117**: 919-927.

Malik, P.K, K.K. Singhal and R.A. Siddique. 2012. Mitigation strategies for enteric methane emission with special reference to biological approaches: a review. *Indian J. Anim. Sci.*, **82**(8): 794-804.

Malik, P.K. and K.K. Singhal. 2008. Influence of supplementation of wheat straw based total mixed ration with saponins on total gas and methane production in vitro. *Indian J. Anim. Sci.*, **78**(9): 987-990.

Malik, P.K., K.K. Singhal, N.M. Soren, A. Thulasi, L. Jose and S.S. Santosh. 2013. Effect of graded level Egyptian clover (*Trifolium alexandrium*) supplementation on in vitro methane production and fermentation pattern. *Indian J. Anim. Sci*., **83**(8): 802-805.

Mao, H.L., J.K. Wang, Y.Y. Zhou and J.X. Liu. 2010. Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of Growing lambs. *Livestock Sci.*, **129**: 56-62.

Mohini, M. and G.P. Singh. 2001. Methane production on feeding Jowar fodder based ration in buffalo calves. *Indian J. Anim. Nutr*., **18**: 204-209.

Navas-Camacho, A., M.A. Laredo, A. Cuesta, H. Anzola and J.C. Leon. 1993. Effect of supplementation with a tree legume forage on rumen function. *Liv. Res. Rural Dev.*, **5**(2).

Ranade, C.D.R. and R.V. Gadre. 1988. Media, p. 67-77. *In Microbial Aspects of Anaerobic Digestion: Laboratory Manual*, Pune: MACS Research Institute.

Singh, G.P. 1998. Methanogenesis and production of greenhouse gases under animal husbandry system. Report of *AP Cess fund project*, National Dairy Research Institute, Karnal, India.

Srivastava, A.K and M.R. Garg. 2002. Use of sulfur hexafluroide tracer technique for measurement of methane emission from ruminants. *Indian J. Dairy Sci.*, **55**(1): 36-39.

Wang, J.K., Y. Jun-An and J.X. Liu. 2011. Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance-a review. *Trop. Anim. Health Pro*., DOI 10.1007/s11250-011-9960-9968.

Wina, E., S. Muetzel and K. Becker. 2005. The impact of saponins or saponin-containing plant materials on ruminant production-A review. *J. Agric. Food Chem.*, **53**: 8093-8105.

Yosioka, I., A. Inada and I. Kitagawa. 1974. Soil bacterial hydrolysis leading to genuine aglycone—VIII : Structures of a genuine sapogenol protobassic acid and a prosapogenol of seed kernels of Madhuca longifolia L. *Tetrahedron*, **30**(6): 707-714.