Indian Journal of Animal Sciences **81** (8): 859–863, August 2011

Effect of monensin and anthraquinone supplementation on rumen fermentation and methane mitigation *in vitro*

S K SIROHI[1], NEHA PANDEY[2], MADHU MOHINI[3], A K PUNIYA[4], B SINGH[5], S S KUNDU[6] and HARSHALA THUBE[7]

National Dairy Research Institute, Karnal, Haryana 132 001 India

Received: 5 September 2010; Accepted: 22 December 2010

ABSTRACT

The effects of addition of monensin sodium and anthraquinone on rumen fermentation and methane production with 3 diets having different roughage concentrate ratio (60:40, 50:50, and 40:60), roughage wheat straw and normal farm concentrate (20% CP and 70% TDN) was studied along with 4 levels of monensin sodium i.e. 0, 10, 15 and 20 ppm and of 9,10, anthraquinone i.e. 0, 20, 40 and 80ppm respectively. The effect of these compounds was studied after 24 h incubation (39±1°C). Monensin and anthraquinone showed positive effect on methane reduction. Microbial biomass yield significantly increased in both treatments in all 3 diets. Similar trend was noticed in ammonia nitrogen. Propionate production increased with the increasing dosage of monensin with decreased A/P ratio. Protozoal population reduced significantly by monensin sodium and anthraquinone supplementation. IVDMD values remained unchanged with monensin whereas anthraquinone supplementation increased it significantly in most of treatment combination.

Key words: Anthraquinone, Ionophores, Methanogenesis

The rate of CH_4 production from livestock is influenced by several factors, including the level of feed intake, type of dietary carbohydrate, feed processing, dietary addition of lipids or ionophores, and changes in ruminal microbial flora and microflora (NRC 2002). Ruminal methanogenesis results in the loss of up to 12% of gross energy intake for forage and up to 4% for concentrate fed cattle (Johnson and Johnson 1995). Inhibition of methanogenesis in the rumen may be useful by increasing the efficiency of energy used in the rumen. Ionophores are antimicrobial compounds that are commonly used in the diets of different species of livestock for increasing the feed conversion efficiency and prevention of bloat diseases. Benefits of feeding ionophores to lactating dairy cows include a shift in the acetate to-propionate ratio towards more propionate and an associated decrease in methanogenesis (Russell and Houlihan 2003), and many other effects. The common examples of ionophores are monensin, lasalocid, laidlomycin, salinomycin and narasin etc**.** Similarly anthraquinones has also shown its effect on methanogens or methane production. *In vitro* evidence has shown that 9,10 anthraquinone can partially inhibit methane (Garcia-Lopez *et al*. 1996) in ruminal fermentations. Therefore, the present study was planned to investigate the effect of monensin sodium and 9,10-anthraquinone on rumen fermentation pattern and methane production in wheat straw based diets in buffalo rumen liquor.

Present address**:** Senior Scientist[1] (e mail; sirohisk @gmail.com),[2]SRF (e mail: neha.ambk@gmail.com), [3]Principal Scientist (e mail: madhummd@gmail.com), Dairy Cattle Nutrition Division; [4,5,6]Principal Scientist (e mails: [4,5,6]**:** e mail: puniya1234 @rediffmail.com; bhupiplp @mailcity.com; kundu.shiv @gmail.com; [7]SRF harshala.thube@gmail.com), Dairy Microbiology Division.

MATERIALS AND METHODS

Preparation of diets: To evaluate the effect of monensin and anthraquinone 3 diets were prepared by taking different roughage concentrate ratio of 60:40, 50:50 and 40:60. The roughage part composed of wheat straw (70 parts) and sorghum (30 parts) or berseem (30 parts) and the concentrate part composed of *Zea maize*, 33%; GNC, 21%; mustard cake, 12%; wheat bran, 20%, deoiled rice bran, 11%; mineral mixture, 2% and salt, 1%. Monensin and anthraquinone were added in incubation medium to achieve final concentration of 0, 10, 15 and 20 ppm and 0, 20, 40 and 80 ppm respectively. All the treatment combinations were arranged in randomized block design with 3 replicates. A set was incubated devoid of substrate with or without monensin and anthraquinone which served as blanks for particular treatment.

Preparation of inoculum: Rumen liquor was collected from a fistulated male buffalo maintained on a standard diet (60 parts roughage: 40 parts concentrate) before morning feeding into a pre-warmed insulated flask and brought into the laboratory. The rumen liquor was filtered through 4 layers

of muslin cloth and then the required amount of filtered rumen liquor used as a source of inoculum.

In vitro gas production: The substrate was milled to pass through 1 mm sieve and 200±10 mg was weighed in glass syringes of 100 ml capacity. The incubation medium was prepared as per Menke and Steingass (1988). The 30 ml incubation medium was dispensed anaerobically in each syringe. Syringes were incubated in shaking water bath at 39±1°C for 24 h as per Menke method. Before incubation, monensin and anthraquinone solutions were prepared, required quantity of monensin sodium and anthraquinone was dissolved in absolute ethanol (99.9%) and diluted to a concentration that each milliliter contains required dose, injected in individual syringes separately by 1 mm syringe into 100 ml syringes. A similar amount of ethanol was added to untreated (control) and separate blanks containing only rumen liquor were also incubated and values were corrected from treatment and control combinations. Syringes were pre warmed (39±1° C) prior to the addition of 30 ml buffered rumen liquor into each syringe under CO_2 flushing. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. Syringes were closed using clamps and were incubated for 24 h in case of *in vitro* dry matter digestibility (IVDMD), total volatile fatty acids (TVFAs), individual volatile fatty acids (IVFAs) and methane estimation.

Total gas production and methane estimation: After 24 h incubation, total gas production was estimated by the displacement of piston during incubation. The gas produced due to fermentation of substrate was calculated by substracting gas produced in blank syringe (containing no substrate, but only the inoculum and buffer) from total gas produced in the syringe containing substrate, inoculum and buffer. For methane estimation, representative gas was sampled from the headspace of syringe in an airtight syringe and injected into Nucon-5765 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q. The gas flow rates for nitrogen, hydrogen and air were 30, 30 and 300 ml/min, respectively. Temperature of injector oven, column oven and detector were 40, 50 and 50° C, respectively. A 50/50 mixture of methane and carbon dioxide was used as a standard.

Partitioning factor (PF) and microbial biomass yield (MBM): The PF was calculated as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor as suggested by Blummel *et al.* (1997).

Microbial mass = Substrate truly degraded - (gas volume × stoichiometrical factor)

where, the stoichiometrical factor used was 2.25.

Estimation of ammonia nitrogen: The supernatant of each syringe including that of blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (2 ml) and steam passed on this using N analyzer and the NH_3 evolved was collected in boric acid solution having mixed indicator and titrated against N/100 H_2SO_4.

Total volatile fatty acid (TVFA) estimation: TVFA concentration (mmol/100 ml) in the supernatant was estimated according to method of Barnett and Reid (1957).

Individual volatile fatty acid (IVFA) estimation: At the end of incubation (24 h) 1 ml of the supernatant was treated with 25% meta-phosphoric acid (4 ml) and kept for 3–4 h at ambient temperature (Erwin *et al.*1961). Thereafter, it was centrifuged at 3000 rpm for 10 min and clear supernatant was collected and stored at –20° C until analyzed. IVFA estimated using gas chromatograph equipped with flame ionization detector (FID) and stainless steel column (length 4'; o.d ¼''; i.d 3 mm) packed with cromosorb 101. Temperature of injection port, column and detector was set at 200, 180 and 210°C, respectively. The flow rate of carrier gas (nitrogen) through the column was 40 ml/min, and the flow rate of hydrogen and air through FID was 30 and 300 ml/min. respectively. Sample (2 ml) was injected through the injection port using Hamilton syringe (10 ml). Individual VFAs of the samples were identified on the basis of their retention time and their concentration (mmol) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values.

Protozoa counting: For protozoal count 1 mm of the fermentation fluid was diluted with 1 ml of formalin (18.5% formaldehyde) and 3–4 drops of brilliant green and then incubated for 24 h at room temperature. The stained protozoa were diluted (if needed) and counted by haemocytometer as per method described by Dehority (1984).

In vitro *true DM degradability*: True DM degradability of feed sample of each syringe containing residues after incubation was estimated as per method given by Van Soest *et al.* (1991).

Proximate analyses and cell wall constituents: The proximate analysis of substrate was carried out as per AOAC (1995). The cell wall constituents of substrates were determined according to methods suggested by Goering and Van Soest (1970).

Statistical analyses: Experimental data of different parameters was analyzed in randomized block design with 3 replicates for analysis of variance (Snedecor and Cochran 1980). The effect of different treatments compared with control were tested using complete randomized block design in OPSTAT statistical software developed by Sheoran (2010).

RESULTS AND DISCUSSION

Results of chemical composition of different diets were presented in Table 1. As expected the CP percentage increased as level of concentrate in the diet increased and reversal was

observed in case of fibre fractions. Results indicated that the monensin supplementation had a positive effect on methane reduction in all 3 diets; the inhibition of methane is higher in high fibre diet as compared to medium and low fibre. This may be due to apparently reduction in DM digestibility and increase in propionate production in high fibre diet. Similarly the reduction in methane production was greater in 15 ppm monensin supplementation i.e. from 36.39 to 24.86, 36.87 to 24.50 and 31.57 to 29.61 ml/g of substrate in diet 1,2 and 3 respectively (Fig. 1). It was suggested that the decrease in methanogenesis is not due to a direct effect of monensin on methanogenic bacteria (Van Nevel and Demeyer 1977), but rather results from a shift in the bacterial population from gram+ve to gram–ve organisms, with a concurrent shift in the fermentation from acetate to propionate as reported by Chen and Wolin (1979). In contrary to the methane reduction with monensin, anthraquinone supplementation resulted in increased methane production with the increasing concentration of anthraquinone in the high fibre diet but decreased in the medium and low fibre diet (Fig.3). The negative effect of anthraquinone on methanogenesis was also observed by Kung *et al.* (2003) but other workers (Garcia-Lopez *et al*.1996) reported positive effect with supplementation of this compund. Monensin supplementation showed unchanged IVDMD values whereas with anthraquinone supplementation IVDMD increased significantly ($P<0.05$) in most of treatment combinations (Tables 2, 3). In contrast reduction in IVDMD after monensin supplementation was reported by Wallace *et al.* (1981).

Microbial biomass yield significantly increased with both monensin and anthraquinone supplementation in all 3 diets. Similar trend was noticed in ammonia nitrogen. Ammonia nitrogen significantly increased in all diets and the differences among diets were statistically significant in both treatment groups. Protozoal population reduced significantly ($P<0.05$) by monensin sodium supplementation and the highest decrease was noticed at highest concentration of monensin i.e. 20 ppm. Also protozoal population was seemed to reduce

Table 1. Chemical composition of different diets (percentage on DM basis)

Parameter	Diet 1 (high fibre)	Diet 2 (medium fibre)	Diet 3 (low fibre)
OM	86.76	87.84	87.56
CP	10.86	12.53	14.27
EE	2.34	3.04	3.48
NDF	62.31	60.45	53.87
ADF	37.20	32.95	29.87
HC	25.11	27.50	24.00
Cellulose	25.66	21.80	19.90
ADL	5.10	5.06	5.36
Total ash	13.24	12.16	12.44
Starch	11.47	13.18	17.44

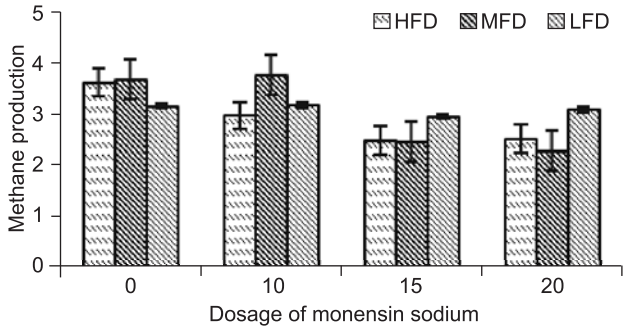

Fig.1. Effect of monensin sodium supplementation on methane production in different diets.

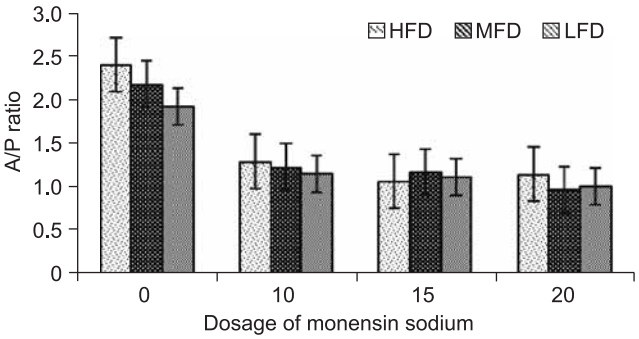

Fig. 2. Effect of monensin sodium supplementation on A/P ratio in different diets.

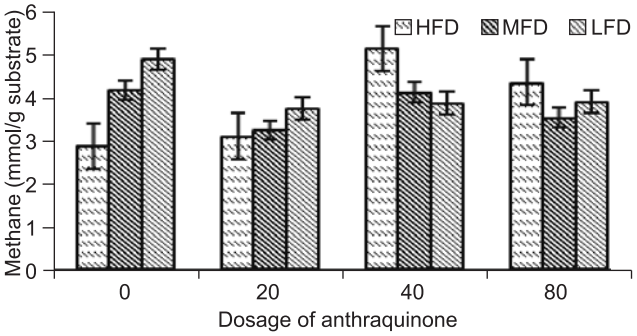

Fig. 3. Effect of 9, 10, anthraquinone supplementation on methane production in different diets.

with the increasing concentration of anthraquinone. Similar decrease in protozoal population was reported by Guan *et al.* (2006). Dennis and Nagaraja (1986) conducted short-term *in vitro* and *in vivo* trials in which a reduction of total protozoa numbers was observed, when monensin or lasalocid were added to ruminal fluid. A study by Rogers *et al.* (1997) showed that extent of reductions in total ruminal protozoa numbers as a result of monensin supplementation decreased with time for animals fed a high-concentrate diet. Ushida and Jouany (1996) reported apparent daily CH_4 emission per

Table 2. Rumen fermentation parameters as affected by monensin sodium supplementation in different diets

Parameters	Dosage of Monensin (ppm)				
	Control	10	15	20	SEM
High fibre diet (60R:40C)					
IVDMD (%)	64.70	58.50	58.50	58.70	NS
PF	2.99	4.21	4.63	4.01	0.12
MBM yield (mg)	31.63	54.56	60.19	51.69	3.72
NH_3-N (mg/100ml)	16.90	16.90	18.90	19.60	1.38
Protozoa ($\times 10^5$cells/ml)	2.00	1.50	1.00	0.50	0.20
Acetic acid (mmol/l)	78.00	52.00	39.00	35.00	2.40
Butyric acid (mmol/l)	8.00	6.00	3.00	2.00	0.50
Propionic acid (mmol/l)	32.00	41.00	36.00	31.00	1.30
A / P ratio	2.42	1.30	1.07	1.15	0.10
Medium fibre diet (50R:50C)					
IVDMD (%)	69.50	66.30	67.50	73.80	NS
PF	2.55	4.34	5.14	3.93	0.12
MBM yield (mg)	16.38	63.88	75.94	63.12	3.72
NH_3-N (mg/100ml)	18.20	18.90	21.00	19.60	1.38
Protozoa ($\times 10^5$cells/ml)	2.50	2.00	1.00	0.50	0.20
Acetic acid (mmol/l)	59.00	39.00	33.00	37.00	2.40
Butyric acid (mmol/ l)	8.00	4.00	2.00	4 .00	0.50
Propionic acid (mmol/ l)	27.00	31.00	28.00	38.00	1.30
A / P ratio	2.19	1.23	1.17	0.97	0.10
Low fibre diet (40R : 60C)					
IVDMD(%)	86.20	84.20	80.50	81.30	NS
PF	3.39	4.64	4.38	4.51	0.12
MBM yield (mg)	58.31	86.94	78.31	81.50	3.72
NH_3-N (mg/100ml)	18.90	23.80	24.50	27.85	1.38
Protozoa ($\times 10^5$cells/ml)	4.00	3.50	2.00	1.50	0.20
Acetic acid (mmol/l)	51.00	34.00	31.00	28.00	2.40
Butyric acid (mmol/l)	6.00	4.00	1.00	3.00	0.50
Propionic acid (mmol/l)	26.00	30.00	27.00	28.00	1.30
A / P ratio	1.93	1.16	1.12	1.01	0.10

*SEM , Standard error of means ; PF, partition factor; MBM , microbial biomass yield.

Table 3. Rumen fermentation parameters as affected by 9,10, anthraquinone supplementation in different diets

Parameters	Dosage of 9,10, anthraquinone (ppm)				
	Control	20	40	80	SEM
High fibre diet (60R:40C)					
IVDMD (%)	63.25	64.75	66.5	67.75	1.09
PF	4.76	4.36	4.54	5.08	0.35
MBM yield (mg)	67.32	61.32	65.16	76.12	NS
NH_3-N (mg/100ml)	12.60	14.70	11.90	14.00	0.53
Protozoa ($\times 10^5$cells/ml)	1.00	1.00	0.50	0.25	0.12
Acetic acid (mmol/l)	42.50	46.80	47.10	46.20	NS
Butyric acid (mmol/l)	0.90	0.80	1.00	0.70	NS
Propionic acid(mmol/l)	19.70	12.40	13.60	13.00	NS
A / P Ratio	2.16	3.80	3.45	3.56	NS
Medium fibre diet (50R:50C)					
IVDMD (%)	65.00	67.25	67.50	69.00	1.09
PF	4.93	4.82	5.22	5.00	0.35
MBM yield (mg)	75.08	75.41	79.99	77.66	NS
NH_3-N (mg/100ml)	11.90	14.70	16.10	15.40	0.53
Protozoa ($\times 10^5$cells/ml)	1.00	0.50	0.25	0.25	0.12
Acetic acid (mmol/l)	42.60	42.50	43.50	41.40	NS
Butyric acid (mmol/l)	0.90	0.80	0.90	0.90	NS
Propionic acid (mmol/l)	13.30	13.50	14.10	14.30	NS
A / P ratio	3.23	3.16	3.13	2.92	NS
Low fibre diet (40R:60C)					
IVDMD (%)	68.50	67.50	72.50	70.75	1.09
PF	4.75	5.34	4.54	4.29	0.35
MBM yield (mg)	75.78	79.08	62.12	71.28	NS
NH_3-N (mg/100ml)	12.60	15.40	12.60	11.90	0.53
Protozoa ($\times 10^5$cells/ml)	0.43	0.40	0.33	0.30	0.12
Acetic acid (mmol/l)	38.50	35.30	31.60	32.90	NS
Butyric acid (mmol/l)	0.80	0.70	0.30	0.80	NS
Propionic acid (mmol/l)	12.30	12.30	14.90	14.80	NS
A / P ratio	3.15	2.91	2.11	2.22	NS

protozoan cell to range from a trace amount to 2 nmol for i*n vitro* systems.

Propionate production increased (Pd"0.05) whereas other VFAs i.e. acetic and butyric acid production reduced with the increasing dosage of monensin with resultant decrease in A/P ratio in all 3 diets (Fig.2). Overall, it is observed that decrease in individual fatty acids i.e acetic acid and butyric acids in monensin supplemented treatment combinations may be due to some energy diverted towards microbial synthesis. Similar results were reported by Richardson *et al.* (1976). Randall *et al.* (1978), Nagaraja (1995), Garcia-Lopez *et al.* (1996), Guan *et al.* (2006) concluded that monensin caused expected changes in microbial fermentation patterns (a decrease in acetate and an increase in propionate, decreased A/P ratio). The increase in propionate production was not reported with anthraquinone but increase in A/P ratio was observed (Fig. 4).

On comparing the effects of both compounds (monensin and anthraquinone) on 3 diets, it was seen that the effect of monensin sodium was more on methane reduction and rumen

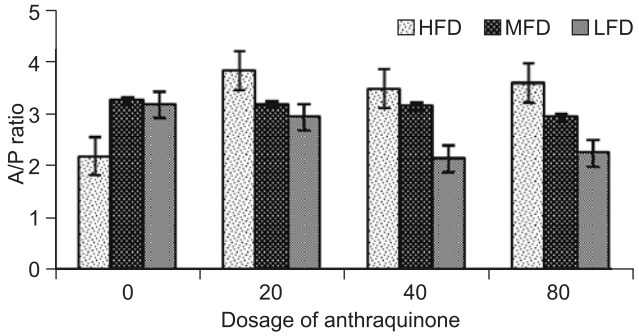

Fig. 4. Effect of 9, 10, anthraquinone supplementation on A/P ratio in different diets.

fermentation than anthraquinone. This decrease appeared to be related to a transitory decline in ciliate protozoa numbers in ruminal fluid. Nonetheless, ionophores should be included in the list of mitigation strategies because of the benefits provided through improved feed use.

9,10-anthraquinone has the ability to partially inhibit methane production under *in vitro* microbial fermentations.

This compound caused changes in the fermentation end products that were similar to findings with other classical methane inhibitors. Further research is required to establish the long-term efficacy of anthraquinones to inhibit methanogenesis and to improve animal performances.

ACKNOWLEDGEMENTS

Authors are thankful to DBT, Ministry of Science & Technology, Govt. of India for providing funds to carry out this work.

REFERNCES

AOAC. 1995. *Official Methods of Analysis.* 16th edn. Association of Official Analytical Chemists, Washington.

Barnett A J and Reid R L. 1957. Studies on the production of volatile fatty acids from grass by rumen liquor in an artificial rumen. *International Journal of Agricultural Science Cambridge* **48**: 315–21.

Blummel M, Makkar H P S, and Becker K. 1997. *In vitro* gas production: a technique revisited. *Journal of Animal Physiology and Animal Nutrition* **77**: 24–34.

Chen M and Wolin M J. 1979. Effect of monensin and lasalocid sodium on the growth of methanogenic and rumen saccharolytic bacteria. *Applied Environmental Microbiol*ogy **38**: 72–77.

Dehority B A. 1993. *Laboratory Manual for Classification of Rumen Ciliate Protozoa.* CRC Press, Boca Raton, F L.

Dehority B A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Applied Environmental Microbiol*ogy **48**:182–85.

Dennis S M and Nagaraja T G. 1986. Effect of lasalocid, monensin and thiopeptin on rumen protozoa. *Research in Veterinary Sc*iences **41**: 251–56.

Erwin E S, Marco G J and Emery E M. 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *Journal of Dairy Sci*ence **44**: 1768–71.

Garcia-Lopez P M, Kung L, Jr and Odom J M. 1996. *In vitro* inhibition of microbial methane production by 9,10-anthraquinone. *Journal of Animal Science* **74**: 2276–84.

Goering H K, and Van Soest P J. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). *Agriculture Handbook No.* 379. ARS, USDA, Washington, D C.

Guan G, Wittenberg K M, Ominski K H and Krause D O. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. *Journal of Animal Science* **84**: 1896–1906.

Johnson K A and Johnson D E. 1995. Methane emissions from cattle. *Journal of Animal Science* **73**: 2483–92.

Kung L, Smith K A, Smagala A M, Endres K M, Bessett C A, Ranjit N K and Yaissle J. 2003. Effects of 9,10 anthraquinone on ruminal fermentation, total-tract digestion, and blood metabolite concentrations in sheep. *Journal of Animal Science* **81**: 323–28.

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

Nagaraja T G. 1995. Ionophores and antibiotics in ruminants. *Biotechnology in Animal Feeds and Animal Feeding*. pp. 173–204, (Eds) Wallace R J and Chesson A V. C H Press, Weinheim.

NRC. 2002. The scientific basis for estimating air emissions from animal feeding operations. *Nutrition* **46***:* 131–48. National Academy Press, Washington, D C.

Randall W, Maanen V, Joseph H, Herbein A, Dare McGilliard and Jerry W Young. 1978. Effects of monensin on *in vivo* rumen propionate production and blood glucose kinetics in cattle. *Journal of Nutrition* **108**: 1002–07.

Richardson LF, Raun A P, Potter E L, Cooley C O and Rathmacher R P. 1976.Effect of monensin on rumen fermentation *in vitro* and *in vivo*. *Journal of Animal Science* **43**: 657–64.

Rogers M, Jouany J P, Thivend P and Fontenot J P. 1997. The effect of short-term and long-term monensin supplementation, and its subsequent withdrawal on digestion in sheep. *Animal Feed Science Technol*ogy **65**:113–27.

Russell J B and Houlihan A J. 2003. The ionophore resistance ruminal bacteria and its potential impact on human health. *FEMS Microbiological Rev*iew **27**: 65–74.

Sheoran O P. 2010. Online Statistical Analysis (OPSTAT) developed by CCS Haryana Agricultural University, Hissar, Haryana, India. (Retrieved from: http://hau.ernet.in/sheoranop/)

Snedecor G W and Cochran W G. 1980. *Statistical Methods.* 6th edn. Iowa State University Press, Ames.

Ushida K and Jouany J P. 1996. Methane production associated with rumen ciliate protozoa and its effect on protozoa activity. *Letter Applied Microbiol*ogy **23**:129–32.

Van Nevel C J and Demeyer D I. 1977. Effect of monensin on rumen metabolism *in vitro*. *Applied Environmental Microbiol*ogy **34**: 251–57.

Van Soest P J, Robertson J B and Lewis B A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**: 3583–97.

Wallace R J, Czerkawski J W and Breckenridge G. 1981. Effect of monensin on the fermentation of basal rations in the Rumen Simulation Technique (Rusitec). *British Journal of Nutrition* **46**: 131–48.