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In vitro Methanogenesis, Microbial Profile and Fermentation of Green Forages with Buffalo Rumen Liquor as Influenced by 2-Bromoethanesulphonic Acid

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ABSTRACT : The interaction of fibre degrading microbes and methanogens was studied using two forages, lucerne (*Medicago sativa*) hay and maize (*Zea mays*) hay, as substrate and 2-bromoethanesulphonic acid (BES) as an additive in an *in vitro* gas production test. Gas and methane production (ml/g dry matter) were significantly higher ($p<0.05$) on lucerne as compared to maize hay. Inclusion of BES in the incubation medium significantly suppressed methane emission irrespective of substrate. The population density of total bacteria, fungi, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* was higher, whereas that of methanogens was lower with maize hay as compared to lucerne as substrate. BES suppressed methanogen population by 7 fold on lucerene and by 8.5 fold on maize at 24 h incubation as estimated by real time-PCR. This suppression was accompanied by almost complete (>98% of control) inhibition of methanogenesis. The proportion of acetate decreased, whereas that of propionate increased significantly by inclusion of BES, resulting in narrowing of acetate to propionate ratio. *In vitro* true digestibility (IVTD) of lucerne was significantly higher as compared to maize but BES inclusion did not affect IVTD. (**Key Words :** 2-Bromoethanesulphonic Acid, Methane Inhibition, Buffalo Rumen Liquor, Real-time PCR, *Fibrobacter succinogenes, Ruminococcus flavefaciens*)

INTRODUCTION

Methanogenesis in the rumen is an essential metabolic process which keeps the fermentation on by continuously utilizing hydrogen released on fermentation of carbohydrates, but this process results in a significant loss of dietary energy (Johnson et al., 1991). In addition to that methane has high global warming potential and therefore enteric emission of methane by the livestock affects the atmosphere adversely (IPPC 1996). Formation of methane from carbondioxide and hydrogen by the rumen methanogens is a multi-step metabolic process involving several enzymes and methyl coenzyme M reductase terminates the reduction process. Halogenated methane analogues like chloroform, carbontetrachloride and methyl chloride are some compounds which inhibit methanogenesis by blocking the terminal enzyme of methanogenesis. Bromoethanesulphonic acid is a halogenated methane analogue and inhibition of methane emission by its inclusion has been demonstrated by Tomkins and Hunter (2003) and Choi et al. (2004). Denman et al. (2005) reported 1.72 fold decrease in methanogen population along with 30% reduction in methane emission by Brahman crossbred steers fed on diet supplemented with bromochloromethane, an halogenated compound without affecting dry matter (DM) intake and live weight gain. The present experiment has been designed to understand the interaction of methanogens with methanogenesis and fibre degrading microbial population due to inhibition of methanogenesis by inclusion of BES in *in vitro* gas production test on two nutritionally different but commonly used green forages.

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MATERIALS AND METHODS

In vitro gas production test

Two fistulated adult buffaloes were fed on a diet of concentrate mixture and wheat straw in 1:1 ratio. The wheat straw contained 909 g organic matter (OM); 31 g crude protein (CP); 15 g ether extract (EE); 835 g neutral detergent fibre (NDF) and 535 g acid detergent fibre (ADF) per kg of DM and the concentrate mixture (consisting of maize, 320 g; solvent extracted soybean meal, 200 g; wheat

bran, 450 g; mineral mixture, 20 g and salt, 10 g/kg) contained 895 g OM; 195 g CP; 40 g EE; 385 g NDF and 164 g ADF per kg of DM. The rumen liquor was collected from these two animals just before feeding (0 h), transported to the laboratory under anaerobic conditions and pooled in equal proportions. Air dried lucerne and maize hay was milled to pass through 1 mm sieve and 200 mg was weighed in graduated glass syringes (100 ml capacity). The incubation medium comprising of buffer and rumen liquor in 2:1 ratio (Menke and Steingass, 1988), was dispensed (30 ml) anaerobically in each syringe as described earlier (Kumar et al., 2007). The control syringes were without 2-bromoethanesulphonic acid sodium salt (BES) and in test syringes 5 mM of BES was added. A similar set of syringes was run separately for estimation of IVTD. Total gas and methane were estimated at 0, 4, 8, 12 and 24 h post inoculation. The other parameters like NH_3-N, enzyme activity, volatile fatty acids and IVTD were analysed at 24 h of incubation. For DNA extraction sampling was done at 0 and 24 h post inoculation.

Estimation of gas, methane and volatile fatty acids

The gas production was recorded periodically by displacement of piston in the syringe. For methane estimation at 24 h, a sample of 100 µl from the gas phase of each syringe was injected into gas chromatograph (NUCON 5765, AIMIL, New Delhi, India) equipped with stainless steel column packed with Porapak-Q and flame ionization detector (FID) using a calibration gas (Spancan, Spantech Products Ltd., England) consisting of 50% methane and 50% CO_2. Flow rate of nitrogen, hydrogen and air were kept as 20, 30 and 320 ml/min and the oven, injector and detector temperatures were 40°C, and 50°C, respectively. For volatile fatty acids estimation, 1.0 ml of fermented medium from each syringe was mixed with 0.2 ml of 25% metaphosphoric acid and after 2 h, centrifuged at 5,000×g for 5 min to get clear supernatant. The supernatant (1 µl) was injected into Nucon-5765 gas chromatograph equipped with flame ionization detector and column packed with cromosorb 101 as described by Cottyn and Boucque (1968). Flow rate of nitrogen, hydrogen and air were kept as 30, 30 and 320 ml/min and the column oven, injector and detector temperatures were 172°C, 270°C and 270°C, respectively.

Estimation of ammonia nitrogen and enzymes

Ammonia nitrogen in the syringe content was estimated as per method of Weatherburn (1967). For estimation of enzyme activities, the fermented medium was extracted with lysozyme and carbontetrachloride (Hristov et al., 1999), sonicated and centrifuged at 24,000×g for 15 min. The supernatant was used for estimation of carboxymethyl cellulase (CMCase) and xylanase activities, using carboxymethylcellulose and xylan as substrates (Agarwal et al., 2000). The protein content of the sample was analysed (Lowry et al., 1951) and enzyme activity was expressed as µmol reducing sugar released per mg protein (IU/mg protein).

Estimation of IVTD

For the estimation of IVTD, the content of syringe was transferred quantitatively to spoutless beaker by repeated washing with 100 ml neutral detergent solution. The content was refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade G1). The DM of the residue was weighed and IVTD of feed was calculated as follows (Van soest and Robertson, 1988).

$$\text{True digestibility (TD)} = \frac{(\text{DM of feed taken for incubation} - \text{NDF residue}) \times 100}{\text{DM of feed taken for incubation}}$$

Real time-PCR

The relative quantification of different microbial groups (bacteria, fungi, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and methanogens) was done with real time PCR using bacteria as the house keeping gene. DNA was isolated from the rumen liquor as sampled above. The cell lysis was done by repeated freezing (liquid nitrogen) and thawing in the presence of Zirconium beads followed by treatment with guanidine thiocyanate and ethanol (IAEA 2004). The primer sets used for RT-PCR were f 5'-CGG CAACGAGCGCAACCC-3', r 5'-CCATTGTAGCACGTG TGTAGCC-3' targeting 16S rRNA gene for rumen bacteria, f 5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3', r 5'-CAAATTCACAAAGGGTAGGATGATT-3' targeting a portion of internal transcribed spacer 1 (ITS1) region for rumen fungi, f 5'-CGAACGGAGATAATTTGAGTTTA CTTAGG-3', r 5'-CGGTCTCTGTATGTTATGAGGTATTA CC-3' for *Ruminococcus flavefaciens*, f 5'-GTTCGGAAT TACTGGGCGTAAA-3', r 5'-CGCCTGCCCCTGAAC TATC-3' for *Fibrobacter succinogenes* and f 5'-TTC GGTGGATCDCARAGRGC-3', r 5'-GBARGTCGWAW CCGTAGAATCC-3' primer set targeting *mcrA* gene for rumen methanogens (Denman and McSweeney, 2005). PCR reaction was carried out in real time PCR machine (Mx 3000P model, Stratagene) using SYBR green QPCR master mix (Stratagene cat # 600548). The reaction was programmed as denaturation at 95°C for 10 min, followed by 40 cycles of 30 sec each for denaturation at 95°C, annealing at 60°C and extension at 72°C. The amplified product specificity was determined by dissociation curve obtained by the cycle of 2 min at 95°C, 15 sec at 60°C and 15 sec at 95°C. The product size verification was also done by agarose gel electrophoresis to confirm the specificity of primers. The results were presented as changes in microbial

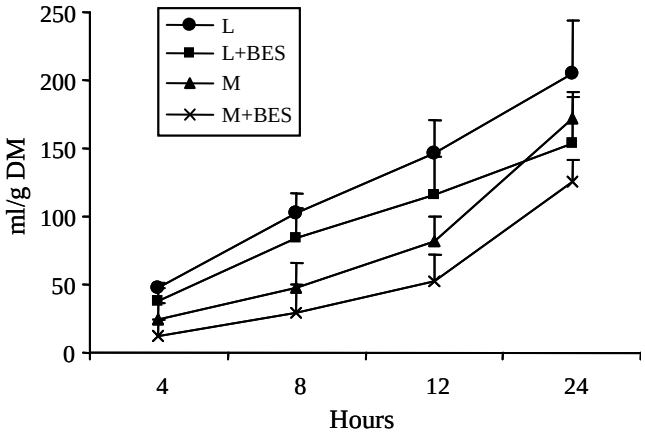

Figure 1. Effect of bromoethanesulphonic acid on *in vitro* gas production (L-lucerne, M-maize).

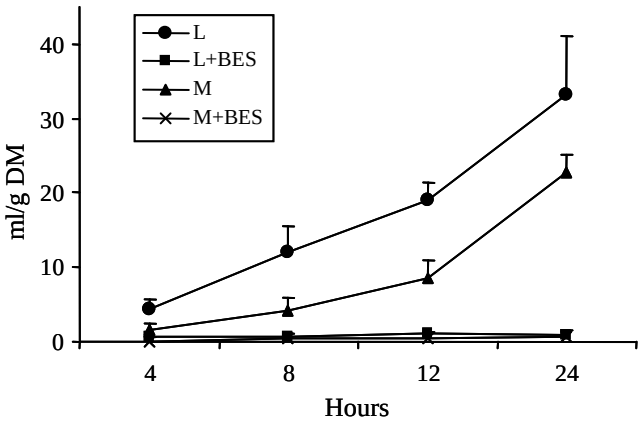

Figure 2. Effect of bromoethanesulphonic acid on *in vitro* methane production (L-lucerne, M-maize).

population relative to control (0 h) taken as one.

Proximate analyses

The DM (ID number 930.15), OM and ash (ID number 942.05), CP (N×6.25, ID number 954.01) and EE (ID number 920.39) of substrates were determined by AOAC (1995) procedures. NDF (estimated without amylase and sodium sulphite and expressed inclusive of ash), ADF (also expressed as inclusive of ash) were analyzed according to the methods described by Van Soest et al. (1991).

Statistical analyses

The data were analyzed using Generalized Linear model ANOVA procedures and Duncan's multiple range test of SPSS (1996).

RESULTS AND DISCUSSION

Effect on gas and methane production

Relatively lower content of CP and higher contents of ADF and NDF were indicated in maize hay compared to lucerne hay. The gas and methane production was significantly higher ($p<0.001$) on lucerne than that on maize hay, both in the presence or absence of methane inhibitor, BES. This might be due to higher digestible fibre content in lucerne hay. The production of gas and methane gradually increased with increase in incubation time irrespective of substrate and the presence of BES (Figure 1). The lower methane content (ml/100 ml) in the fermentation gases in the initial stages of incubation might be due to slower growth of methanogens as compared to sugar fermenting bacteria, which were responsible for total gas production. Methane in fermentation gases was significantly higher on lucerne hay than that on maize hay (16.31% vs. 13.19%) after 24 h of incubation. Total methane generated at 24 h incubation was also significantly higher on lucerne hay than that on maize hay as substrate (Figure 2). In the presence of 2-bromoethanesulphonic acid the methanogenesis was almost completely stopped (>98% inhibition) on both the substrates tested in the present experiment. The previous studies also indicated that 2-bromoethanesulphonic acid at a concentration of 5×10^{-5} M inhibited methanogenesis by 86% and increased hydrogen production several folds (from 0.5 μmol/min to 24.5 μmol/min), but had no effect ($p>0.05$) on volatile fatty acid production or proportion of different acids (Sauer and Teather, 1987). In another study Martin and Macy (1985) reported that BES (30 μM) inhibited methanogenesis by 76% and hydrogen accumulation was increased 20 folds. Choi et al. (2004) reported a significant reduction in *in vitro* methane production by BES without affecting *in vitro* feed degradability and the reduction was enhanced by adding malate along with BES. Tomkins and Hunter (2003) reported a reduction in methane energy from 3.9 to 0.6% of gross energy intake without affecting daily weight gain in feedlot steers fed on BES, but rumen microbes gradually adapted to it.

Effect on VFA production

The production of volatile fatty acids was significantly higher on lucerne hay as substrate in comparison to that on maize hay (Table 1) which might be due to better degradability of lucerne and higher availability of cell contents as indicated by the chemical composition of the two feeds. On maize hay the acetate:propionate (A/P) ratio was significantly lower ($p<0.05$) as compared to lucerne hay. Inclusion of BES reduced TVFA and A/P ratio irrespective of substrate. According to Russel (1998) and Lila et al. (2005), methane production was highly correlated with acetate: propionate ratio in the rumen. Decreased A/P ratio accompanied with inhibition in methanogenesis by halogenated analogue has also been demonstrated in other laboratories (Trei et al., 1971; Johnson et al., 1972; Saravanan, 2000).

Table 1. Effect of 2-bromoethanesulphonic acid (BES) on rumen metabolites, enzyme activities and *in vitro* true digestibility (IVTD) of feed at 24 h of incubation

Parameters	Diet				SEM	Significance (p value)		
	Lucerne hay		Maize hay			Diet	Treatment	D×T
	- BES	+ BES	- BES	+ BES				
TVFA (mg/100 ml)	3.30	3.08	2.68	2.47	0.054	<0.001	0.007	0.955
Acetate (%)	76.62	74.14	78.68	76.91	0.600	0.007	0.016	0.684
Propionate (%)	17.31	19.01	16.08	18.82	0.413	0.233	<0.001	0.384
Butyrate (%)	6.08	6.84	5.30	4.30	0.391	0.005	0.835	0.121
Ac/Pr	4.45	3.98	4.80	4.27	0.126	0.080	0.007	0.844
NH_3-N (mg/100 ml)	30.74[a]	29.69[b]	29.41[b]	26.57[c]	0.240	<0.001	<0.001	0.012
Xylanase (IU/mg protein)	520[c]	502[c]	687[a]	587[b]	9.642	<0.001	<0.001	0.004
CMCase (IU/mg protein)	233	225	352	336	5.374	<0.001	0.127	0.581
IVTD (g/g DM)	0.565	0.578	0.284	0.285	0.802	<0.001	0.526	0.614

L = Lucerne hay; M = Maize hay.
[a, b, c] Means with different superscripts in a row differ significantly.

Table 2. Effect of feed and 2-bromoethanesulphonic acid (BES) on relative quantification of different microbial groups at 24 h against 0 h (considering as 1.0) by real time PCR

Feed	Bacteria	Methanogen	Fungi	*R. flavefaciens*	*F. succinogens*
Lucerne hay					
-BES	57.40	12.12	0.14	0.05	0.82
+BES	2.39	1.72	0.06	0.55	0.37
Maize hay					
-BES	12.70	1.11	1.64	0.68	2.25
+BES	10.60	0.13	2.37	0.92	3.25

IVTD

IVTD of both the substrates increased with the incubation time and the digestibility of lucerne hay was higher than that of maize hay (Table 1) due to lower fibre content. But the activities of CMCase and xylanase were significantly higher with maize as compared to lucerne. The results of the present experiment corroborate with earlier findings (Kamra et al., 2003; Agarwal et al., 2004) where the *in sacco* digestibility was higher in case of low fibre diet and activities of both the fibre degrading enzymes were higher in higher fibre diet. Inclusion of BES did not affect IVTD of both the substrates, which might be due to no effect of BES on fibre degrading microbial populations and CMCase activity in the incubation medium, though there was a significant reduction in xylanase activity. In steers also there was a 30% reduction in methane production by using bromochlromethane as feed additive, but fibre digestibility was not affected (Denman et al., 2005).

Microbial profile

The number of total bacteria was higher on lucerne hay than that on maize hay as substrate. With lucerne as substrate, total bacterial population increased by 57.4 folds at 24 h incubation as compared to that at 0 h but on addition of BES the population size was 2.34 folds higher than that at 0 h. With maize the increase of bacterial population was 12.7 times and on BES inclusion in the incubation medium it reduced to 10.6 times of the 0 h population. The fungal counts in the incubation medium were higher on maize hay than that on lucerne hay. After 24 h incubation with maize hay there was 1.64 and 2.37 folds increase without and with BES. Similarly the number of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* was higher on maize hay as compared to that on lucerne hay. This might be due to significantly higher contents of ADF and NDF in maize hay. However, methanogens were higher in lucerne hay as compared to maize hay which might be due to higher cell contents and better digestibility of lucerne hay. BES inhibited methanogen population by 7 times in lucerne hay and 8.5 times in maize hay, respectively. The microbial profile on the two forages supported the previous findings of the authors' laboratory in which high fibre in ration supported higher fibrolytic enzymes activity and the number of fibre degrading microbes in the rumen liquor of buffaloes (Kamra et al., 2003; Agarwal et al., 2004). With Lucerne hay as substrate the fibre degrading microbes like fungi, *R. flavefaciens* and *F. succinogenes* were lower than those with maize hay as substrate, reflecting the dependence of microbial profile on chemical composition of the substrate. The results also indicated that BES specifically hampered the activity of methanogens. Denman et al. (2005) demonstrated a 30% reduction in methane emission along with two fold decrease in methanogen population by feeding of bromochloromethane supplemented diet in steers without disturbing fibre digestibility indicating no adverse effect on fibre degrading microbes. Wina et al. (2005) studied interaction of methanogens, *Fibrobacter* sp., *R. albus*, *R. flavefaciens* and rumen fungi as influenced by

feeding of methanol extract of *Sapinus rarak*. There was depression in all the populations studied except *Fibrobacter* sps. indicating variable response of different fibre degrading microbial populations to the feed additive.

CONCLUSIONS

The chemical composition of feed plays an important role in the microbial and enzyme profile of feed fermented *in vitro* with buffalo rumen liquor. High fibre content in diet supports higher numbers of fibre degrading bacteria, fungi and fibre degrading enzyme activities while high digestible fibre content might be responsible for larger number of methanogens. Inhibition of methanogenesis with BES results in a significant decrease in the number of methanogens, but has no adverse effect on digestibility of feed. The data also indicate that the response of rumen microbes to the feed additives is also regulated by the type of feed.

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