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Effect of sulphate and blend of plant parts containing secondary metabolites on *in vitro* methanogenesis, digestibility and feed fermentation with buffalo rumen liquor

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ABSTRACT

A blend of plant parts containing fruit of *Phyllanthus emblica* (amla), seed of *Foeniculum vulgare* (fennel) and seed of *Trachyspermum ammi* (ajwain) mixed in equal proportion (BP) was tested at 0, 10 and 20% of the substrate along with 0, 2.5 and 5% of sodium sulphate (S) for their effects on *in vitro* methane production and feed fermentation. Inclusion of combination of BP with S did not affect production of total gas, methane and feed digestibility. *In vitro* gas production was not influenced by any level of S, whereas, it increased linearly with increasing level of BP. Inhibition pattern of methane production (ml/g DM) was similar by inclusion of both S and BP and the inhibition was 21.3% by inclusion of BP20S5. *In vitro* true digestibility of feed was significantly increased by inclusion of both S and BP and was 10.9% higher with BP20S5 as compared to control (BP0S0). Total volatile fatty acids were not affected by any of the treatments, whereas, acetate was increased and propionate and butyrate were significantly reduced resulting in increased acetate to propionate ratio. Ammonia production was not affected by inclusion of neither S nor BP. It may be summarized that the blend of amla, ajwain and fennel and sodium sulphate can be explored as feed additive to mitigate methane production with an additional benefit of improvement in feed digestibility.

Key words: Buffalo rumen liquor, *In vitro* true digestibility, Methane, Plant secondary metabolites, Sulphate

Rumen harbours billions of interactive microbes like bacteria, archaea, protozoa, fungi, bacteriophages and mycoplasma in its anaerobic environment which has capability to ferment poor quality lignocellulosic feeds into various end products. The end products of ingested feeds are volatile fatty acid (VFAs), ammonia (NH_3), carbon dioxide (CO_2) and methane (CH_4). These VFAs and NH_3 are used as energy and protein sources, whereas, gases (CO_2 and CH_4) are released in the environment. Both CO_2 and CH_4 are potent greenhouse gases responsible for global warming (IPCC 2007). Methane has 23 times more global warming potential than CO_2. That is how livestock is contributing to the environmental pollution. Methanogenesis also decreases the efficiency of feed utilization in the animals, as 2–12% of gross energy of feed consumed by the animals wasted in the form of methane (Johnson *et al.* 1993, Kamra *et al*. 2012, Innus *et al*. 2015) leads to huge economic loss for livestock sector. In India, buffaloes contribute about 42.8% of total enteric methane; hence can be considered as one of the major contributors to global warming (Patra 2014). Now, due to climate change, eco-friendly and precision feeding practices are adopted in animal feeding to minimize methane production and avoid wastage of nutrients to maximize the animal production. The goal of mitigation of methane production can be achieved either by directly attacking the methanogen population or by using energetically more efficient terminal electron acceptors as a feed supplement (Hook *et al.* 2010). In rumen, sulphate reducing bacteria (SRB) are present which have better affinity with hydrogen than methanogens have. The SRBs reduce sulphate to hydrogen sulphide by utilizing ruminal hydrogen but, main hurdle is its limited activity due to low sulphur concentration and low number of SRB in the rumen (Bal and Ozturk 2006, Caldwell *et al.* 2008). The hydrogen sulphide produced by these bacteria can be utilized by most of the other microbes which cannot use sulphate directly. Sulphide is also involved in the synthesis of S containing amino acids; therefore inclusion of sulphate in the diet improves the growth of rumen microbes which ultimately results in improved performance of animals.

Plant secondary metabolites (PSM) such as saponins, tannins, lignins, alkaloids, essential oils etc. are natural components of herb and spices and have antimethanogenic as well as antiprotozoal activity (Sakthivel *et al.* 2012, Yatoo

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et al. 2014, Kamra *et al.* 2015, Samal *et al.* 2016a,b). Therefore, the study was conducted to evaluate the potential of sulphur (as inorganic terminal electron acceptors) and plant parts rich in secondary metabolites individually and in combinations to inhibit methanogenesis and stimulate fibre digestion.

MATERIALS AND METHODS

Experimental design: The plant parts used in this study were fruit of *Phyllanthus emblica* (amla), seed of *Foeniculum vulgare* (fennel) and seed of *Trachyspermum ammi* (ajwain). Sodium sulphate (S) was used as a source of sulphate. The plant parts (amla, fennel and ajwain) were dried, powdered and mixed in equal proportion to prepare a blend (BP), which was used at the rate of 0, 10 and 20% along with sodium sulphate (S) at the rate of 0, 2.5 and 5% of the substrate resulting in total nine treatments viz, BP0S0, BP0S2.5, BP0S5, BP10S0, BP10S2.5, BP10S5, BP 20S0, BP20S2.5, BP20S5.

In vitro *gas production test: In vitro* gas production tests were conducted as per the procedure of Menke and Steingass (1988). The substrate used (200 mg/syringe) was wheat straw and concentrate mixture in 1:1 ratio and pooled rumen liquor collected from two fistulated buffaloes fed on diet containing wheat straw and concentrate mixture in equal proportion. The concentrate mixture consisted of maize grain 32%, de-oiled soyabean meal 20%, wheat bran 45%, mineral mixture 2% and salt 1%. Incubation medium (30 ml) was dispensed anaerobically in each syringe. One set of syringes comprised each treatment in triplicate and two blank syringes (without substrate) and three such sets were run for each blend, hence three replicates for each treatment. The syringes were incubated for 24 h at 39°C, thereafter, syringes were withdrawn from the incubator and all the analyses were done.

Proximate analyses: The dry matter (DM), crude protein (CP), ether extract (EE) of substrate (wheat straw and concentrate mixture), BP were estimated by AOAC (1995). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analyzed according to Van Soest *et al.* (1991).

Estimation of gas and methane production: Gas production was estimated after 24 h of incubation by piston displacement. Net gas produced by feed fermentation was calculated by subtracting from total gas produced in blank. For methane estimation, 100 µl of gas sampled from headspace of calibrated syringe was injected into Nucon-5765 gas chromatograph equipped with PorapakQ column and flame ionization detector (Agarwal *et al.* 2008). A mixture of 50% carbon dioxide and 50% methane was used as standard.

Estimation of metabolites: For VFA estimation, 0.5 ml fermented medium was mixed with 0.1 ml of 25% metaphosphoric acid and allowed to stand at room temperature for 1 h. The mixture was centrifuged at 10,000 rpm for 10 min and 1 µl of the supernatant was injected on Nucon-5765 gas chromatograph equipped with chromosorb 101 column and FID as per the procedure described by Cottyn and Boucque (1968) with some modifications (Agarwal *et al.* 2008). Fermented medium was analyzed for NH_3-N (Weatherburn 1967).

Statistical analysis: The statistical analyses of the data obtained were done using factorial (3×3) univariate ANOVA with contrast analysis using the model, intercept + BP + sulphate + BP × sulphate to analyze the effect of BP, S and their interaction and means were compared using Tukey's test if the main effect was significant ($P<0.05$) using SPSS Version 16.0.

RESULTS AND DISCUSSION

The proximate compositions of concentrate mixture, wheat straw, and blend of BP used in *in vitro* gas production test (IVGPT) are presented in Table 1. The crude protein content of concentrate mixture, wheat straw and BP was 19.79, 3.24 and 11.13%, respectively.

In vitro gas production (ml/g DM) was not affected by the inclusion of S, whereas, it increased linearly by increasing the level of BP in the incubation medium irrespective of level of S (Table 2). The maximum increase was 23.39% with BP20 as compared to control (BP0S0). Methane production (ml/g DM) was significantly inhibited by inclusion of both S and BP independently and there was no interaction between the two (Table 2). The methane inhibition was 10.16 and 11.47% with S5 and BP20. The IVTD of feed increased significantly with S5 and BP20 and the increase was 10.94% by inclusion of BP20S5 in the incubation medium (Table 2). TVFA was not affected either by inclusion of S or BP, however, propionate and butyrate decreased significantly by inclusion of BP resulting in increased acetate to propionate ratio. Ammonia nitrogen remained unaffected both with S and BP (Table 2).

The plant parts used in the present study are the rich source of essential oils (ajwain and fennel) and tannins (amla). Amla is also a rich source of vitamin C and exhibit antioxidant characteristics. Inclusion of essential oils or tannins inhibits gas and methane production (Pawar *et al.* 2014, Yatoo *et al.* 2014) in dose dependent manner. Several *in vitro* studies have suggested that the effect of thymol (active principle of ajwain) is diet and pH dependent (Cardozo *et al.* 2005, Castillejos *et al.* 2006). In the present study, the gas production was increased with inhibition in methane production to a maximum of 11.47% which was

Table 1. Proximate composition of feed and blend of plant parts

Attribute (%)	Concentrate mixture	Wheat straw	BP
Dry matter	94.09	93.84	90.30
Organic matter	89.15	90.40	88.71
Crude protein	19.79	3.24	11.13
Ether extract	3.58	1.69	5.31
Neutral detergent fibre	27.44	78.12	44.89
Acid detergent fibre	11.97	49.55	23.40

Table 2. Effect of sodium sulphate (S) and BP on production of total gas, methane and rumen metabolites

S	BP (% of substrate)			Mean	SEM	P Value		
	BP (0)	BP (10)	BP (20)			BP	S	BP*S
				Total gas (ml/gm DM)				
S0.0	131.70	148.24	162.23	147.39	3.53	<0.001	0.937	0.993
S2.5	131.11	151.50	163.13	148.58				
S5.0	132.58	150.02	162.53	148.38				
Mean	131.80^{Z}	149.92^{Y}	162.63^{X}					
				Methane (ml/gm DM)				
S0.0	24.60	20.95	20.59	22.05^{A}	0.61	<0.001	<0.002	0.257
S2.5	22.00	21.02	19.56	20.86^{AB}				
S5.0	20.64	19.44	19.37	19.81^{B}				
Mean	22.41^{X}	20.47^{Y}	19.84^{Y}					
				IVTD (%)				
S0.0	57.31	60.55	61.28	59.71^{B}	0.76	0.03	<0.001	0.344
S2.5	61.19	63.03	62.16	62.13^{A}				
S5.0	62.79	62.58	63.58	62.98^{A}				
Mean	60.43^{Y}	62.05^{XY}	62.34^{X}					
				TVFA (mM)				
S0.0	46.90	44.79	46.69	46.13	5.44	0.75	0.95	0.99
S2.5	41.23	44.71	47.38	44.44				
S5.0	42.77	43.32	48.57	44.87				
Mean	43.63	44.27	47.53					
				Acetate (%)				
S0.0	71.79	73.10	73.07	72.65	0.22	<0.001	0.323	0.60
S2.5	71.66	72.85	73.14	72.55				
S5.0	71.38	72.36	73.22	72.32				
Mean	71.61^{Y}	72.77^{X}	73.14^{X}					
				Propionate (%)				
S0.0	20.01	19.64	19.80	19.82	0.21	0.001	0.07	0.33
S2.5	20.74	19.67	19.62	20.01				
S5.0	20.83	20.25	19.83	20.31				
Mean	20.53^{X}	19.85^{Y}	19.75^{Y}					
				Butyrate (%)				
S0.0	8.20	7.27	7.13	7.53	0.19	<0.001	0.69	0.38
S2.5	7.59	7.48	7.24	7.44				
S5.0	7.79	7.34	6.95	7.37				
Mean	7.86^{X}	7.38^{Y}	7.10^{Y}					
				A:P ratio				
S0.0	3.59	3.73	3.69	3.67	0.04	<0.001	0.09	0.41
S2.5	3.47	3.71	3.73	3.64				
S5.0	3.43	3.58	3.70	3.57				
Mean	3.49^{Y}	3.67^{X}	3.71^{X}					
				NH_3-N (mg/dl)				
S0.0	10.30	10.24	11.55	10.70	0.23	0.188	0.988	0.038
S2.5	10.57	11.79	9.97	10.77				
S5.0	9.88	10.07	12.19	10.71				
Mean	10.25	10.69	11.24					

BP, Blend of amla, ajwain and fennel in equal proportion; S, Sodium sulphate. ^{AB}Different superscript in a column differ significantly. ^{XYZ}Different superscript in a row differ significantly.

less as compared to other *in vitro* studies where inhibition in methane production reached up to 99% with pure essential oils or extracts (Pawar *et al.* 2014, Yatoo *et al.* 2014, Arif *et al.* 2015). But the inhibition in methane production was associated with decreased IVTD except few which showed no effect at low levels (Pawar *et al.* 2014). In the present study, since plant parts were used in powder form, therefore, the secondary metabolites did not reach up to the level which could be detrimental for feed fermentation rather were beneficial in terms of methane inhibition associated with increased IVTD. *In vitro* inclusion or feeding of sulphur has resulted in reduction in methane production confirming its role as alternate electron acceptor (Van Zijderfeld *et al.* 2010, Phuong *et al*. 2011). No effect on TVFA along with increased acetate to propionate ratio was also observed in many of *in vitro* studies (Patra *et al.*

2009, Samal *et al.* 2016a, Perme *et al.* 2016) indicating that methane inhibition is not always related to increased propionate production because different plant secondary metabolites have different mode of action.

The study showed that the BP and sulphur could be used as feed additive to mitigate methane production and to improve feed digestibility. Further evaluation of the feed additive by conducting feeding trials is required to validate their potential.

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