



Effect of feeding a mixture of plants containing secondary metabolites and peppermint oil on rumen fermentation, microbial profile and nutrient utilization in buffaloes

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ABSTRACT

A mixture of plants (mix) and peppermint oil (PO) were evaluated in fistulated buffaloes in 3×3 latin square design to study their effect on rumen fermentation, microbial profile, microbial protein synthesis and nutrients utilization. The plant mixture consisted of leaves of mango (*Mangifera indica*), jamun (*Eugenia jambolana*), guava (*Psidium guajava*), seed pulp of harad (*Terminalia chebula*) and fennel (*Foeniculum vulgare*) in equal proportions and was fed @ 40 g (dried) /100 kg BW, while peppermint oil was supplemented @ 2 ml/100 kg BW. There was no difference in post-prandial changes in pH and total volatile fatty acids in rumen liquor among groups. The proportion of propionate was significantly lower in mix as compared to control and PO supplemented groups. The ammonia nitrogen (mg/dl) was lower in mix fed group as compared to control, whereas, total-N, TCA precipitable-N, activities (IU/mg protein) of carboxy-methylcellulase, xylanase and acetylestrase and protozoa population were similar in all the groups. Real time PCR studies indicated an increase of about 9- and 7-folds in total bacteria and *Fibrobacter succinogenes* populations and a 30-fold decrease in *Ruminococcus flavefaciens* population with no change in fungal population by mix supplementation. The PO supplementation decreased about 7- and 1.5- folds in fungal and *R. flavefaciens* populations, whereas about *F. succinogenes* population showed 6-fold increase as compared to control. The concentrations of purine derivatives in urine were similar in all the groups reflecting similar microbial protein synthesis. The dry matter, TDN and DCP intakes and nutrients digestibility, except that of ether extract were not affected by the feed additives. These results indicated that dietary supplementation of mix and peppermint oil at the present level had no adverse effect on rumen fermentation pattern, microbial protein synthesis and nutrient digestibility in buffaloes.

Key words: Buffalo, Digestibility, Plant secondary metabolites, Purine derivatives, Rumen fermentation

The use of antibiotics or chemicals as feed additives to improve livestock productivity is primarily discouraged due to their residues in the livestock product and adverse effects on the rumen microbes. The plants rich in secondary metabolites (PSM) appear to be the ideal replacement of chemical feed additives (Teferedegne 2000) which are safe for animal as well as for the human being who are the ultimate consumer of livestock products. Therefore, a series of *in vitro* experiments were conducted to evaluate the plants rich in secondary metabolites as rumen modifier (Kamra *et al.* 2006,

2008, Patra *et al.* 2006, 2009, Agarwal *et al.* 2006, 2009, and Kumar *et al.* 2010). The present experiment was conducted using mixture of leaves mango, jamun and guava (rich in tannins and essential oils), fennel (essential oils) and harad (tannins) as feed additive to study its effect on nutrient utilization, rumen fermentation, enzyme and microbial profile.

MATERIALS AND METHODS

Animal feeding and sample collection: Three adult male buffaloes (*Bubalus bubalis*) fitted with rumen fistula (average b. wt. 387.06±18.26 kg) were used in a 3×3 latin square design. The animals were fed to meet their maintenance requirement as per Mandal *et al.* (2003) in such a way that the dry matter intake through concentrate mixture and wheat straw was maintained in 1:1 ratio in all groups. The composition of the concentrate mixture was maize, 55; soybean meal, 12; wheat bran, 30; mineral mixture, 2 and

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salt, 1 parts. The additive comprised dried powder of leaves of mango (*Mangifera indica*), jamun (*Eugenia jambolana*), guava (*Psidium guajava*), and seed pulp of harad (*Terminalia chebula*) and fennel (*Foeniculum vulgare*) in equal proportions. The 3 treatments were control, plants mix @ of 40 g/100 kg BW and peppermint oil @ 2 ml/100 kg BW, respectively. Each feed additive was offered by mixing it with concentrate mixture daily. Wheat straw was offered after the consumption of concentrate mixture. Water was offered twice a day, and during sample collection period, water was given after 0 h collection and after last collection. Daily feed intake for each animal was recorded. After 21 d of feeding, sampling of rumen liquor was done for 3 consecutive days at 0, 2, 4, 6 and 8 h post feeding. For biochemical analyses, the samples were pooled animal wise daily. For microbial and enzyme profile, rumen contents were collected at 0 h post feeding. For purine derivatives estimation, an aliquot from 24 h urine collection was taken and pH was adjusted to 3.0 by 10% H₂SO₄ and kept at -20°C till analysed. The urine collection was also done for 3 consecutive days.

Digestion trials: For conducting metabolism trial, animals were kept in metabolic cages for 4 days prior to actual collection to acclimatize them to the new surroundings. The collection of samples of feed offered, residue left and faeces voided in 24 h was done after proper mixing for 6 d. Dried feed and faecal samples were ground (1 mm) and used for proximate and fiber composition analysis.

Analyses of rumen metabolites, enzyme activities and microbial profile: The pH of strained rumen liquor was recorded immediately after collection. Total volatile fatty acids and its fractions were estimated with gas chromatograph equipped with flame ionization detector and chromosorb 101 column (Cottyn and Boucque 1968) with some modifications (Agarwal *et al.* 2008). The rumen liquor samples were analyzed for ammonia nitrogen (Weatherburn 1967), total nitrogen and TCA precipitable nitrogen (AOAC 1995). Non protein nitrogen (NPN) was calculated as difference of total nitrogen and TCA precipitable nitrogen of rumen liquor.

The enzymes were extracted from the whole rumen content by treating the sample with lysozyme and carbon tetrachloride (Hristov *et al.* 1999) followed by sonication. After centrifugation at 10 000×g at 4°C for 30 min, the clear supernatant was collected for enzyme estimation. The activities of carboxy-methylcellulase (Endo-β-1,4-D-glucanase, EC 3.2.1.4) and xylanase (Endo-β-1,4-D xylan xylanohydrolase, EC 3.2.1.8) were estimated as per Agarwal *et al.* (2000) and acetyl esterase (acetic-ester acetylhydrolase, EC 3.1.1.6) activity was determined according to Huggins and Lapides (1947). Protein content of the enzyme samples was estimated by Lowry *et al.* (1952). Unit of enzyme activity for carboxy-methylcellulase and xylanase activity was defined as mmol reducing sugar min⁻¹ ml⁻¹ and for acetyl esterase as mmol p-nitrophenol min⁻¹ ml⁻¹. Enzyme activity was expressed as specific activity (units/mg protein).

Ciliate protozoa were counted under the microscope (Kamra *et al.* 1991). The relative quantification of different microbial groups (total fungi, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) was done by real-time PCR using total bacteria as the house keeping gene and the specific primers for different microbial groups (Denman and McSweeney 2005, Agarwal *et al.* 2008). DNA was extracted from the rumen liquor by repeated freezing (liquid nitrogen) and thawing in the presence of Zirconium beads followed by treatment with guanidine thiocyanate and ethanol (IAEA 2004). The amplification of DNA was carried out in real time PCR machine using SYBR green QPCR master mix and programmed as denaturation of DNA 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.

Urine analyses for purine derivatives: Urine was filtered (0.22 μm Millipore filter) and diluted (1:5) with distilled water and analysed for allantoin (Young and Conway 1942), uric acid and creatinine using commercial kit. Purine derivatives (PD) were calculated as follows:

$$PD \text{ (mmol/L)} = \text{Allantoin (mmol/L)} + \text{uric acid (mmol/L)}.$$

Purine derivatives:creatinine (PDC) index = [PD/creatinine] × W^{0.75} where, W^{0.75} is the metabolic body weight (kg) of the animal.

Proximate and fibre analyses of feed and faeces: The feed and faecal samples were analysed for DM, OM and ash, CP (N×6.25) and EE as per AOAC (1995). NDF was estimated without amylase and expressed inclusive of ash as per Van Soest *et al.* (1991) and ADF (also as inclusive of ash).

Statistical analyses: All statistical analyses were performed using SPSS (1996) computer package. For comparison of multiple groups, Generalized Linear model ANOVA procedures and Duncan's multiple range tests were used.

RESULTS AND DISCUSSION

The pH of rumen liquor was not affected, irrespective of the feed additives, and varied within normal range indicating that there was no adverse affect on rumen environment (Table 1). The concentration of total volatile fatty acids and the proportion of acetate and butyrate were similar in all the groups, whereas, the proportion of propionate decreased significantly (P<0.05) resulting in a significant increase in acetate to propionate ratio in plant mix supplemented group (Table 1). Patra *et al.* (2007) also did not find any change in rumen pH following the supplementation of harad, garlic and a mixture of both in buffaloes. Fernandez *et al.* (2005) found similar VFA pattern after addition of a specific blend of essential oil compounds in a semi-continuous flow fermenter. Ammonia nitrogen

Table 1. Effect of dietary supplementation of mix and peppermint oil on rumen metabolites, enzyme activities and purine derivatives

Parameter	Control	Mix	Peppermint oil	SEM
pH	6.77	6.70	6.72	
<i>Rumen metabolites</i>				
Ammonia nitrogen (mg/100 ml)	16.36 ^{ab}	14.40 ^b	19.80 ^a	0.89
Total nitrogen (mg/100 ml)	74.40	69.51	68.00	2.97
TCA-ppt nitrogen (mg/100 ml)	50.31	57.77	52.66	2.41
TVFA (mmole/100 ml)	6.63	6.62	6.17	0.16
Acetic acid (%)	73.25	74.39	73.14	0.26
Propionic acid (%)	18.01 ^a	17.20 ^b	17.84 ^{ab}	0.14
Butyric acid (%)	8.73	8.39	9.00	0.29
<i>Enzyme activity (IU/mg protein)</i>				
Xylanase	1.34	1.11	1.54	0.12
Carboxymethylcellulase	0.37	0.30	0.45	0.033
Acetylesterase	0.79	0.59	0.77	.078
<i>Purine derivatives in urine</i>				
Creatinine (mM/l)	3.54	3.44	3.04	0.42
Uric acid (mM/l)	0.24	0.22	0.27	0.013
Allantoin (mM/l)	1.35	1.05	1.44	0.071
Creatinine (μ M/kgW ^{0.75} /day)	530.4	474.2	559.3	58.71
Allantoin (μ M/kgW ^{0.75} /day)	292.6	224.3	266.5	34.04
Uric acid (μ M/kgW ^{0.75} /day)	53.1	46.8	51.0	6.36
PDC index	70.2	58.7	57.8	9.73
<i>Microscopic count of protozoa ($\times 10^4$/ml)</i>				
Holotrichs	0.582	0.672	0.713	0.047
Spirotrichs	26.41	24.59	27.62	1.575
<i>Rumen microbial population considering control as 1.0 (real time-PCR)</i>				
Bacteria	1.00	8.98	3.14	
Fungi	1.00	0.98	0.14	
<i>R. flavefaciens</i>	1.00	0.03	0.62	
<i>F. succinogenes</i>	1.00	7.23	6.12	

concentration in the rumen liquor was significantly lower in mix fed group than in PO fed group but the values were similar to that in control group (Table 1). The low level of ammonia nitrogen might be due to the synergistic effects of different secondary metabolites, like tannins, essential oils, glucoside, steroids etc. in different plant parts of the mix. Patra *et al.* (2007) also reported a significantly ($P < 0.05$) lower level of ammonia nitrogen in garlic fed fistulated buffaloes. No affect of essential oil compounds and PO on ammonia nitrogen was also reported in semi-continuous fermenter and *in vitro* gas production test (Fernandez *et al.* 2005, Agarwal *et al.* 2009). There was no difference in total nitrogen and TCA-ppt nitrogen between control and treatment groups. Supplementation of both additives did not affect activities of fibre degrading enzymes, viz. CMCase, xylanase and

acetylstase. Patra *et al.* (2007) also did not record any affect of harad, garlic and their mixture on CMCase and xylanase activities. However, inclusion of peppermint oil decreased CMCase and xylanase activities in a dose dependent manner.

Microbial profile: The protozoa numbers were similar in all groups as reported by Patra *et al.* (2007) following the supplementation of garlic, harad and their mixture. But with PO, there was a reduction in protozoa population in a dose dependent manner in *in vitro* gas production test (Agarwal *et al.* 2008). Similarly the extracts of leaves of mango, jamun (Kumar *et al.* 2010) and guava (Kamra *et al.* 2006) when added in the incubation medium also demonstrated antiprotozoal activity individually in *in vitro* system. No effect on protozoa population in the present *in vivo* study might be due to lower dose of feed additive. An increase in bacterial and *F. succinogenes* populations by 9- and 3- fold and 7- and 6- fold, on supplementation of mix and PO, respectively, while 7- fold decrease in fungal population on PO supplementation as compared to control. Lower *Ruminococcus flavefaciens* population density in treatment groups than in control indicated that specificity of the plant secondary metabolites towards the rumen microbes. McIntosh *et al.* (2003) demonstrated a sensitivity of rumen anaerobic fungi towards commercial blend of essential oils in an *in vitro* experiment. This might be the reason for a decrease in fungal population with PO. In mix fed animals also there was an essential oil component, but the level of oil might not be sufficient to affect the rumen anaerobic fungi. In *in vitro* system, the rumen microbes (*R. succinogenes* and *F. ruminococcus*) behaved in a different manner when PO was included in the incubation medium (Agarwal *et al.* 2009). The difference in the behavior of the rumen microbes in the 2 experiments might be due to the 2 different systems and the duration of treatment. Busquet *et al.* (2006) and Castillejos *et al.* (2007) were of the opinion that short term batch culture studies are not sufficient for the biological expression of essential oils as it has been observed that in long term *in vitro* trials the rumen bacteria might have adapted to these compounds.

Purine derivatives: Similar values of the purine derivatives in all groups indicated that the feed additives had no adverse effect on microbial protein synthesis. All the values were within normal range as reported by Dipu *et al.* (2006) in buffaloes. Newbold *et al.* (2004) also did not record any influence of a specific preparation of essential oil on microbial protein production in sheep.

Nutrient intake and digestibility: The daily dry matter intake and nutrients digestibility were similar in all groups (Table 2) indicating that the levels of feed additive tested are safe in terms of feed intake. Hosoda *et al.* (2005) reported significantly lower nutrient digestibility in cows following the dietary supplementation of 5% peppermint. In *in vitro* experiment, depression in *in vitro* true degradability accompanied with depression in fibre degrading enzymes

Table 2. Effect of dietary supplementation of plant mix and peppermint oil on dry matter intake and digestibility of nutrients in buffaloes

Parameters	Control	Mix	Peppermint oil	SEM
<i>Dry matter intake (kg/day)</i>				
Concentrate	2.64	2.66	2.65	0.062
Wheat straw	2.54	2.67	2.57	0.095
Total	5.17	5.32	5.22	0.154
DDMI	3.09	3.16	3.17	0.102
TDNI	2.97	3.05	3.00	0.085
DCPI	0.278	279.9	282.9	13.47
<i>Nutrient digestibility (%)</i>				
Crude protein	60.41	60.27	60.38	1.53
Ether extract	67.62 ^b	68.37 ^b	79.70 ^a	3.90
NDF	67.24	65.10	67.94	0.86
ADF	44.55	48.54	50.15	2.08
Hemicellulose	70.97	70.02	70.09	1.77
Cellulose	58.08	60.24	61.83	1.41

a, b values bearing different superscript in a row differ significantly at $P < 0.05$.

activity in a dose dependent manner on inclusion of PO was reported by Agarwal *et al.* (2009). The individual components of the Mix behaved differently in *in vitro* system, like extracts of harad and jamun leaf depressed IVTD (Patra *et al.* 2006, Kumar *et al.* 2010), but fennel and mango leaf extracts had no affect (Patra *et al.* 2009, Kumar 2010). Therefore, no effect of mix feeding might be due to the cumulative effect of the components or due to the 2 different systems or the dose and form of the additives.

Our results indicated that the levels of the feed additives in the diet of buffaloes had no adverse effect on nutrients utilization, rumen fermentation pattern and microbial protein synthesis. Though there was a shift in the fibre degrading microbial populations yet it did not translate into any influence on fibre degradation. The antiprotozoal activity of different ingredients of the mix demonstrated individually in *in vitro* studies was not reflected in this experiment, which might be due to insufficient level of the secondary metabolites (like tannins, glucoside, steroids, essential oils etc.) present in these plant parts used in the mix. Therefore, further studies at different levels of inclusion are needed to optimize the level of feeding for its practical use as a rumen modifier for improving feed utilization in the rumen.

REFERENCES

- Agarwal N, Agarwal I, Kamra D N and Chaudhary L C. 2000. Diurnal variations in the activities of hydrolytic enzymes in different fractions of rumen liquor of Murrah buffalo. *Journal of Applied Animal Research* **18**: 73–80.
- Agarwal N, Kamra D N, Chatterjee P N, Kumar R and Chaudhary L C. 2008. Changes in microbial profile, methanogenesis and fermentation of green forages with buffalo rumen liquor as influenced by 2-bromoethanesulphonic acid. *Asian-Australasian Journal of Animal Science* **21**: 818–23.
- Agarwal N, Kamra D N, Chaudhary L C and Patra A K. 2006. Effect of *Sapindus mukorossi* extracts on *in vitro* methanogenesis and fermentation characteristics in buffalo rumen liquor. *Journal of Applied Animal Research* **30**:1–4.
- Agarwal N, Shekhar C, Kumar R, Chaudhary L C and Kamra D N. 2009. Effect of peppermint (*Mentha piperita*) oil on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. *Animal Feed Science and Technology* **148**: 321–27.
- AOAC. 1995. *Official Methods of Analysis*. 16th edn. Association of Official Analytical Chemists, Washington D C.
- Busquet M, Calsamiglia S, Ferret A and Kamel C. 2006. Plant extracts affect *in vitro* rumen microbial fermentation. *Journal of Dairy Science* **89**: 761–71.
- Castillejos L, Calsamiglia S, Ferret A and Losa R. 2007. Effects of dose and adaptation time of a specific blend of essential oil compounds on rumen fermentation. *Animal Feed Science and Technology* **132**: 186–201.
- Cottyn B G and Boucque C V. 1968. Rapid methods for the gas chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agricultural Food Chemistry* **16**: 105–07.
- Denman S E and McSweeney C S. 2005. Quantitative (real-time) PCR. *Methods in Gut Microbial Ecology for Ruminants*. pp. 105–15. (Eds) Makkar H P S and McSweeney C S. Springer, Netherlands.
- Dipu M T, George S K, Singh P, Verma A K and Mehra U R. 2006. Measurement of microbial protein supply in Murrah buffaloes (*Bubalus bubalis*) using urinary purine derivatives excretion and PDC index. *Asian-Australasian Journal of Animal Science* **19**: 347–55.
- Fernandez M, Lopez S, Rodriguez A B, Garcia Gonzalez R, Frehner M, Gonzalez J S. 2005. Effects of CRINA Reg. additive on ruminal fermentation *in vitro*. *XI Jornadas Sobre Produccion Animal, Zaragoza* 11–12–2005 Vol. I and II, Pp.566–68. Mayo, Spain.
- Hosoda K, Nishida T, Park W Y and Eruden B. 2005. Influence of *Mentha piperita* L. (Peppermint) supplementation on nutrient digestibility and energy metabolism in lactating dairy cows. *Asian-Australasian Journal of Animal Science* **18**: 1721.
- Hristov A N, McAllister T A, Cheng K T. 1999. Effect of diet digesta processing, freezing and extraction procedure on some polysaccharide degrading activities of ruminal contents. *Canadian Journal of Animal Science* **79**: 73–81.
- Huggins C and Lapides J. 1947. Acetyl esters of p-nitrophenol as substrate for the colorimetric determination of esterase. *Journal of Biological Chemistry* **170**: 467–82.
- IAEA. 2004. *qPCR Workshop for Rumen Microbiology Ecology*. International Atomic Energy Agency, Pp 1–38, Australia.
- Kamra D N, Agarwal N and Chaudhary L C. 2006. Inhibition of ruminal methanogenesis by tropical plants containing secondary compounds. *International Congress Series* **1293**: 156–63.
- Kamra D N, Patra A K, Chatterjee P N, Kumar R, Agarwal N and Chaudhary L C. 2008. Effect of plant extracts on methanogenesis and microbial profile of the rumen of buffalo: a brief overview. *Australasian Journal of Experimental Agriculture* **48**: 175–78.
- Kamra D N, Sawal R K, Pathak N N, Kewalramani N and Agarwal N. 1991. Diurnal variation in ciliate protozoa in the rumen of black buck (*Antelope cervicapra*) fed green forage. *Letters in Applied Microbiology* **13**: 165–67.
- Kumar R, Kamra D N, Agarwal N, Chaudhary L C and Zadbuke S

- S. 2010. Effect of tree leaves containing plant secondary metabolites on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. *Animal Nutrition and Feed Technology* (In press).
- Lowry O H, Rosenbrough N J, Farr A R, and Randall R J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–75.
- Mandal A B, Paul S S and Pathak N N. 2003. *Nutrient Requirements and Feeding of Buffaloes and Cattle*. International Book distributing Co., Lucknow, India.
- McIntosh F M, Williams P, Losa R, Wallace R J, Beever D A and Newbold C J. 2003. Effects of essential oils on ruminal metabolism and their protein metabolism. *Applied Environmental Microbiology* **69**: 5011–14.
- Newbold C J, McIntosh F M, Williams P, Losa R and Wallace R J. 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. *Animal Feed Science and Technology* **114**: 105–12.
- Patra A K, Kamra D N and Agarwal N. 2009. Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds *in vitro*. *Journal of Science Food and Agriculture*. (Retrieved from: www.interscience.wiley.com).
- Patra A K, Kamra D N, Agarwal N and Chatterjee P N. 2007. Effects of *Terminalia belerica* and *Allium sativum* as feed additives on rumen fermentation, enzyme activities and microbial populations in Murrah buffaloes. *Indian Journal of Animal Nutrition* **24**: 251–55.
- Patra A K, Kamra D N and Agarwal N. 2006. Effect of plant extracts on *in vitro* methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. *Animal Feed Science and Technology* **128**: 276–91.
- SPSS. 1996. *Statistical Packages for Social Sciences*. version 7.5, SPSS Inc., Illinois, USA.
- Teferedegne B. 2000. New perspective on the use of tropical plants to improve ruminant nutrition. *Proceeding of Nutrition Society* **59**: 209–14.
- Van Soest P J, Robertson J B and Lewis B A. 1991. Methods for fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**: 3583–97.
- Weatherburn M W. 1967. Estimation of ammonia nitrogen by colorimetric method. *Analytical Chemistry* **39**: 971–74.