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Effect of phyto additives and *Saccharomyces cerevisiae* on rumen ferementation and microbial profile in buffaloes

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ABSTRACT

To study the effect of plants containing plant secondary metabolites (PSM) and *Saccharomyces cerevisiae* on rumen ferementation and microbial profile, four fistulated adult buffaloes were fed in 4×4 Latin square design. The four groups were, control, without additive; T1, mixture of harad seed pulp and garlic bulb (2% of DMI); T2, *S. cerevisiae* (350g feremented feed containing *S. cerevisiae* 10^6 /g)*;* T3, mixture of *harad* seed pulp, garlic bulb and *S. cerevisiae*. All the animals were fed on a basal diet consisting of wheat straw and concentrate mixture in 50:50 ratio. Daily DM intake was similar in all the four groups. Feeding of any of the dietary treatments did not affect rumen fluid pH, concentration of ammonia N and lactic acid. The total volatile fatty acids and its fractions except butyrate were also not influenced by supplementation of any of the additives. The activities of ruminal enzymes viz. carboxymethylcellulase, xylanase, avicelase and acetyl esterase were similar in all the four groups. The rumen microbial density of total bacteria, *Ruminococcus flavefaciences*, *R. albus,* methanogens and fungi were not changed whereas *Fibrobacter succinogenes* and protozoa populations were significantly reduced in T1 but were at par of control by inclusion of yeast in the diet. It is concluded that mixture of *harad* and garlic alone or in combination with yeast culture did not influence rumen fermentation however microbial profile (*F. succinogenes* and protozoa) was improved by feeding yeast as additive. The additives tested seem to have potential to alter rumen microbial ecology and can further be explored for its efficacy in improving the performance of the animals.

Key words: Buffalo, Microbial profile, Plant secondary metabolites, Rumen fermentation

Modification of rumen microbial fermentation to decrease ruminal methane and ammonia nitrogen production and ciliate protozoa by using feed additives is a useful strategy to improve production efficiency of ruminant animals (Alexander *et al*. 2008). There is an increasing interest in using herbal products containing plant bioactive compounds instead of chemical compounds as feed additive to modify rumen fermentation for improving feed utilization and productive performance of ruminant animals. The reasons for increasing popularity of PSMs as feed additive in livestock are their natural occurance in plants, are known for their anti-microbial activity and are commonly being used as medicines, spices since ages.

The use of feed additives of plant origin as methane inhibitors have been studied extensively (Sallam *et al.* 2009). Majority of the studies conducted so far, are based on *in vitro* experiments (Kamra *et al.* 2006, Kumar *et al.* 2011) with limited *in vivo* studies (Santos *et al.* 2004). There is a chance of reducing digestibility of the nutrients consumed by the animals and affecting the performance of animals adversely if additives containing PSM are used because these additives might affect the desired microbial population present in the gastrointestinal tract (Wallace 2004). The microbial feed additives especially *S. cerevisiae* are able to improve the intestinal microbial consortia resulting in improved feed utilization and better performance of the animals (Kamra *et al*. 2002, Chevaux and Mazzia-Fabre 2007*)*. On the above background, the present study was conducted to study the effect of using a mixture of plant parts containing secondary metabolites, *S. cerevisiae* and their combination on rumen fermentation, microbial profile of buffaloes. The PSMs sources used in the present study were *harad* seed pulp (source of tannins) and garlic (source of essential oils), already tested in the author' laboratory (Patra *et al*. 2011).

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MATERIALS AND METHODS

Experimental animals, feeding and sampling: Adult male buffaloes (4), average body weight (424 kg) and fitted with a permanent fistula, were used in 4×4 latin square design. The 4 groups were, control, without additive; T1, mixture of *harad* seed pulp and garlic bulb (2% of DMI); T2, *S. cerevisiae* (350g feremented feed containing *S. cerevisiae* 10^6 /g*;* T3, mixture of *harad* seed pulp, garlic bulb and *S. cerevisiae*. The fermented feed was prepared as described by Chaudhary *et al.* (2008). The animals were fed on wheat

straw and concentrate mixture in 50:50 ratios to meet their maintenance requirement as per ICAR (2013). The concentrate mixture contained 25parts maize, 14 parts solvent extracted soybean meal, 58 parts wheat bran, 2 parts mineral mixture and 1part salt. The detailed composition of concentrate mixture is given in Table 1.

The feed additives were mixed with the concentrate mixture and wheat straw was offered after the concentrate mixture was consumed by the animals. In each phase of 30 d duration, after 21 days of feeding rumen liquor was sampled for 2 consecutive days at 0, 2, 4, 6 and 8 h post-feeding, whereas, rumen content was sampled at 0 h feeding. The pH of the rumen liquor was recorded at every collection and then the rumen liquor of every collection was pooled day wise and animal wise. These pooled samples were used for the estimation of rumen metabolites. Rumen contents collected at 0 h from different different approachable corners of the rumen were processed for enzyme estimation immediately. The rumen contents were squeezed and liquid portion was used for DNA extraction used for microbial profile.

Estimation of rumen metabolites

The VFAs concentration in the rumen liquor was estimated using gas chromatograph equipped with chromosorb 101 column and flame ionization detector. The conditions were similar as described by Agarwal *et al*. (2008). The rumen liquor was also analysed for ammonia nitrogen (Weatherburn 1967) and lactic acid concentration (Barker and Summerson 1941).

Estimation of rumen enzymes: For estimation of enzyme activity the rumen contents were subjected to extraction of enzymes with lysozyme and carbontetrachloride (Hristov *et al*. 1999). The activities of carboxymethyl cellulase, avicelase (microcrystalline cellulose) and xylanase were estimated as per Agarwal *et al*. (2000) using carboxymethyl cellulose, avicel and xylan as substrate respectively. Acetyl esterase was estimated as per Huggins and Lapides (1947).

Rumen microbial profile by qPCR: Total genomic DNA from the rumen liquor was extracted as per Yu and Morison (2004). The qPCR was standardized for absolute quantification of microbial cells (bacteria, fungi, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, methanogens and protozoa) per gram of rumen liquor. For preparation of standard curve, the purified PCR product using specific primer was cloned in pGEMT easy vector (Promega) and transformed in *Escherichia coli*. The plasmid with insert was extracted and copy number was calculated (Ritalahti *et al*. 2006). The plasmid was serially diluted and amplified using the same primer and standard curve was plotted for copy number vs ct value. The unknown sample was amplified and copy number was read on standard curve. The amplification reactions were performed in a total volume of 20 µl containing 2 ng of template DNA, 10 µl of 2X Kappa SYBR master mixes, 0.6 µl of each primer (6 pmol) and nuclease free water. The primer sequences and conditions used for real time PCR for various targeted microorganisms are listed in Table 1.

Statistical analysis: The data were statistically analyzed using generalized linear model (GLM) procedures and difference between treatments and periods was analyzed by using analysis of variance (ANOVA) (SPSS 1995). The means were compared by Tukey's Multiple Comparison Test. The difference between means was declared significant at ($P < 0.05$).

RESULTS AND DISCUSSION

Nutrient density of feed and voluntary feed consumption: The chemical composition of the concentrate mixture and wheat straw used for feeding of fistulated buffaloes is given in Table 2. The crude protein content of concentrate mixture and wheat straw was 15.8 and 3.04%, respectively. The composition of all the nutrients matched the nutrient requirement profile of adult buffaloes for maintenance requirement (ICAR 2013). Daily total dry matter intake (DMI) in terms of kg/d and g/kg $W^{0.75}$ was similar ($P>0.05$) in all the 4 groups (Table 2). Wanapat *et al.* (2013) reported that the DMI was not affected by dietary herb

Table 1. Primer sequences and conditions used for real time PCR for various targeted microorganisms

Target organism	Primer sequence	Amplicon size	Annealing temp.	Reference
Bacteria	F 5'-CGGCAACGAGCGCAACCC R 5'-CCATTGTAGCACGTGTGTAGCC	130	60	Denman *et al.* (2005)
Fungi	F 5'-GAGGAAGTAAAAGTCGTAACAAGGTTT R 5'-CCAAATTCACAAAGGGTAGGATGATT	110	60	Denman *et al.* (2005)
Methanogen	F 5'-TTCGGTGGATCDCARAGRGC R 5'-GBARGTCGWAWCCGTAGAATCC	140	60	Denman *et al.* (2007)
R. flavefaciens	F 5'-CGAACGGAGATAATTTGAGTTTACTTAGG R 5'-CGGTCTCTGTATGTTATGAGGTATTA	132	60	Denman *et al.* (2005)
Fibrobacter succinogenes	F 5'-GTTCGGAATTACTGGGCGTAAA R 5'-CGCCTGCCCCTGAACTATC	121	60	Denman *et al.* (2005)
R. albus	F 5'-CCC TAA AAG CAG TCT TAG TTC G R 5'-CCT CCT TGC GGT TAG AAC A-3'	175	55	Koike and Kobayashi (2001)
Protozoa	F 5'-GCTTTCGWTGGTAGTGTATT R 5'-CTTGCCCTCYAATCGTWCT	223	54	Sylvester *et al*. (2004)

Table 2. Chemical compositions (% DM basis) of wheat straw and concentrate mixture fed to fistulated buffaloes

Chemical composition	Concentrate mixture	Wheat straw
Organic matter	91.31	90.79
Crude protein	15.8	3.04
Ether extract	3.43	0.75
Neutral detergent fiber	35.37	80.25
Acid detergent fiber	9.54	50.03
Acid detergent lignin	4.02	11.04
Total ash	8.21	8.01

supplementation (lemon grass powder alone or in combination of peppermint powder or garlic powder) in rumen-fistulated crossbred beef cattle. Feeding of yeast culture also has not affected DMI in Murrah buffaloes (Bhima *et al*. 2014).

Rumen fermentation patterns: The data on the effect of feeding additives on production of rumen metabolites are presented in Table 3. Feeding of any of the additives had no effect (P>0.05) on rumen fluid pH, concentration of ammonia N and lactic acid. Kumar *et al.* (2011) did not observe any affect on rumen pH and ammonia nitrogen by feeding leaves mixture to buffaloes. In agreement with our finding no change in pH was also observed by Beauchemin and McGinn (2006) with feeding of essential oils (Crina ruminants; 1g/d) in cattle. Yang *et al.* (2007) reported that addition of essential oils had no effect on ruminal fluid concentration of ammonia nitrogen. Thao *et al*. (2014) reported no change in ruminal ammonia nitrogen due to eucalyptus (*E. camaldulensis*) crude oils supplementation in swamp buffaloes fed rice straw base diet. Bermingham *et al.* (2001) found decreased ammonia nitrogen in sheep rumen fed sainfoin which contained 38 g CT/kg DM. Molero *et al.* (2004) speculated that the effects of essential oils on nitrogen metabolism might be the result of inhibition of proteolytic activity or a decrease in the attachment and colonization of feed by proteolytic microbes.

The concentration of volatile fatty acids and its molar proportion were not affected (P>0.05) by supplementation of any of the feed additives to the diet of fistulated buffaloes (Table 3). Wanapat *et al*. (2013) reported no change in TVFA concentration and molar proportion of butyrate, however, there was decrease in acetate and increase in propionate proportion in all the three treatment groups as compared to control cattle. Kumar *et al.* (2011) observed no affect of ffeding leaves mixture on TVFA but propionate concentration reduced significantly as compared to control buffaloes. Yeast feeding showed conflicting results on rumen fatty acid concentration. Corona *et al*. (1999) reported that, steers and sheep supplemented with yeast had lower total VFA concentration and molar proportion of butyric acid inin the rumen. In contrast to above study, the ruminal VFA concentration and molar proportions of acetic, propionic and butyric acid was not affected by feeding yeast cultures to fistulated heifers (Kowalik *et al*. 2012).

Rumen enzyme activities: Activity of ruminal enzymes viz. carboxymethyl-cellulase, avicelase, xylanase and acetyl esterase activity were not affected (P>0.05) by addition of plant additives and yeast to the diet of fistulated buffaloes

Table 3. Effect of feeding mixture of herbal plants, *S. cerevisiae* and combination of both on dry matter intake and rumen fermentation in buffaloes

Attributes	Treatment				SEM	Pvalue		
	CON	T1	T2	T3		T	P	T*P
BW (kg)	339.0	355.5	347.0	364.8	12.16	0.912	0.615	0.598
BW (kg $W^{0.75}$)	78.86	81.73	80.25	83.33	2.150	0.917	0..423	0.736
Dry matter intake								
(kg/d)	7.20	7.3	7.43	7.56	0.129	0.813	0.517	0.687
(g/kg $W^{0.75}$)	91.64	89.64	93.13	91.08	1.189	0.813	0.463	0.713
Rumen metabolites								
pH	6.67	6.68	6.69	6.74	0.11	0.93	0.99	0.49
NH_3N (mg/dL)	10.73	9.25	9.95	10.04	0.82	0.42	0.36	0.18
Lactate (mg/dL)	1.28	1.67	1.70	1.71	0.28	0.42	0.63	0.39
TVFA (mg/dL)	7.57	6.30	7.41	6.89	0.85	0.49	0.78	0.85
Acetate (mg/dL)	5.16	4.33	5.14	4.69	0.64	0.56	0.83	0.82
Propionate (mg/dL)	1.46	1.21	1.42	1.36	0.12	0.21	0.46	0.7
Isobutyrate (mg/dL)	0.06	0.04	0.05	0.04	0.01	0.06	0.01	0.48
Butyrate (mg/dL)	0.83	0.70	0.77	0.80	0.12	0.73	0.35	0.85
Isovelarate (mg/dL)	0.04^{b}	0.01^{a}	0.02^{a}	0.01^{a}	0.01	0.00	0.01	0.01
A : P	3.50	3.56	3.61	3.50	0.20	0.92	0.71	0.54

[ab]Means with different superscript in a row differ significantly; VFA, volatile fatty acid; A:P, acetate-propionate ratio; CON, control with no additive; T1, mixture of *harad* seed pulp and garlic bulb (2% of DMI); T2, *S. cerevisiae* (350g feremented feed containing *S. cerevisiae* 10^6 /g*;* T3, mixture of *harad* seed pulp, garlic bulb and *S. cerevisiae*. *;* SEM, standard error of mean.

Table 4. Effect of feeding mixture of herbal plants, *S. cerevisiae* and combinationof both on enzyme activities (U/mg protein) in rumen content of fistulated buffaloes

Enzymes	Treatment				SEM	Pvalue		
	CON	T1	T2	T3		T	P	T*P
CMCase	0.21	0.23	0.25	0.29	0.07	0.72	0.03	0.88
Xylanase	0.53	0.50	0.49	0.56	0.18	0.98	0.06	0.97
Avicelase	0.08	0.10	0.15	0.15	0.03	0.16	0.01	0.1
Acetylesterase	0.43	0.44	0.43	0.49	0.15	0.97	0.06	0.67

Unit, µmol of glucose released/min/ml for CMCase and avicelase; µmol of xylose released/min/ml for xylanase; µmol of p-Nitrophenol released/min/ml for acetyl esterase; CON, control with no additive; T1, mixture of *harad* seed pulp and garlic bulb (2% of DMI); T2, *S. cerevisiae* (350g feremented feed containing *S. cerevisiae* 10^6 /g*;* T3, mixture of *harad* seed pulp, garlic bulb and *S. cerevisiae*. *;* SEM, standard error of mean.

Table 5. Effect of feeding mixture of herbal plants, *S. cerevisiae* and combinationof both on Log^{10} value of microbes in rumen liquor of fistulated buffaloes

Microbes	Treatment				SEM	Pvalue		
	CON	T1	T2	T3		T	P	T*P
Total bacteria	10.32	10.17	10.02	9.70	0.33	0.36	0.95	0.75
F. succinogenes	9.41^{b}	8.74^{a}	9.09^{ab}	9.02^{ab}	0.18	0.05	0.29	0.31
R. flavefaciens	6.55	6.22	6.19	5.75	0.28	0.13	0.62	0.56
R. albus	5.76	5.78	5.37	5.11	0.49	0.51	0.99	0.98
Methanogens	7.33	7.25	6.98	7.04	0.38	0.76	0.97	0.99
Fungi	6.92	6.66	6.70	6.47	0.34	0.64	0.15	0.54
Protozoa	8.74^{b}	8.52^{a}	8.67^{ab}	8.08^{a}	0.10	0.05	0.04	0.19

^{ab}Means with different superscript in a row differ significantly; VFA, volatile fatty acid; A:P: acetate-propionate ratio; CON, control with no additive; T1, mixture of *harad* seed pulp and garlic bulb (2% of DMI); T2, *S. cerevisiae* (350g feremented feed containing *S. cerevisiae* 10^6 /g*;* T3, mixture of *harad* seed pulp, garlic bulb and *S. cerevisiae*. *;* SEM, standard error of mean.

(Table 3). Similarly to this study no effect on CMCase, xylanase and acetyl esterase activity was observed by feeding a combination of plants containing secondary metabolites to fistulated buffaloes (Kumar *et al*. 2011). There are a very few reports on changes in rumen microbial enzyme activity by feeding plant parts containing PSMs. Some *in vitro* studies have showed increase, decrease or no change in enzyme activities by inclusion of extracts of different plant parts with variable PSMs (Patra *et al*. 2006).

Microbial profile: The changes in population density of various rumen microbes as estimated by real time PCR showed that supplementation of the additives did not affect the population of total bacteria, *R. flavefaciences*, *R. albus,* methanogens and fungi where as *F. succinogenes* and protozoa were changed (Table 4).

The population of *F. succinogenes* which is one of the important fibre degrading microbes in rumen was significantly lower ($P<0.05$) in T1 group (where mixture of *harad* and garlic was fed) as compared to control but was similar to T2 and T3 groups where yeast culture was used as microbial feed additive (Table 5). It seems that in T2 and T3 groups, yeast protect these two microbial populations from the adverse effect of *harad* and garlic. In case of the rumen protozoa the effectiveness of *harad* and garlic was not altered when yeast culture was added to this group of animals. The reduction in protozoal population is indirectly indicates the improved rumen function as there is inverse relationship between protozoa and methane production in the rumen. Anantasook *et al*. (2013) reported that population density of total bacterial, *R. albus* and viable proteolytic bacteria were not affected, but that of cellulolytic bacteria, *F. succinogenes, R. flavefaciens* and methanogens were higher, whereas, amylolytic bacteria and protozoa were lower by dietary supplementation of rain tree pod meal. Feeding of leaves meal to buffaloes also resulted in decrease *R. flavefaciens* and increase in total bacteria and *F. succinogenes* (Kumar *et al*. 2011). Protozoa populations were reduced by eucalyptus crude oils supplementation while fungi zoospores, total, amylolytic and cellulolytic bacterial populations and viable bacteria remained the same (Thao *et al*. 2014). There are several factors like type, source, dose, mode of feeding and interaction of PSMs present in a single plant product etc. which affects the response of herbal feed additives.

Our results indicated that the feeding of mixture of harad and garlic alone or in combination with yeast culture did not influence rumen fermentation, microbial enzymes however microbial profile (*F. succinogenes* and protozoa)

was changed. The adverse effect of *harad* and garlic on some rumen microbes like *F. succinogenes* (a key microbe involved in fibre degradation) was reduced by inclusion of yeast culture in the feed. The additives tested seems to have potential to alter rumen microbial ecology and can further be explored its efficacy in improving the performance of the animals.

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