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Effect of High, Medium and Low Grain Proportions on Buffalo Rumen Ecosystem

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ABSTRACT

The aim of this study was to detect the major shifts in fermentation, enzymes activities and microbial communities by feeding diet with high, medium and low grain. Buffaloes were adapted to 3x3 switch over design at 64%, 44% and 23% maize grain in diet fed at 90% of dry matter (DM) intake respectively for 21 days. At the end of each treatment, rumen fluid was collected at 0 h and 4 h after feeding. We performed qPCR based absolute quantification of selected rumen microbes in rumen fluid of buffalo. No significant difference was observed in rumen microbial community and enzymatic activity, however, gas produced (ml/ g DM) and *in vitro* digestibility (IVTD %) were significantly lower ($P<0.05$) in high grain group in comparison with low grain group. Ammonia nitrogen and lactic acid concentrations were significantly ($P<0.01$) higher in high grain than low grain diet. From above experiment we conclude that there are changes in rumen fermentation due to change in grain proportions, which should be closely monitored during the dietary shifts in ruminants.

Key words: Diet, Enzyme, Real time PCR, Rumen, VFA

INTRODUCTION

The complex symbiotic microbiota of the rumen is responsible for breakdown of plant fibre for maintenance and production of ruminants. This microbiota is highly responsive to changes in diet, age, antibiotic use, and the health of the host animal. Further, it varies according to geographical location, season, and feeding. The incorporation of concentrates in ruminant diets is intended to increase the energy, protein, minerals, and vitamins intake of the animal. The feed utilization, productive efficiency, and the fibre digestion may depend on the nature of the concentrate (Morand-Fehr and Sauvant, 1987). However, long-term feeding of a high-concentrate diet causes a decreased ruminal pH value due to the accumulation of volatile fatty acids (VFA) and lactic acid, and, a chronic digestive disorder known as sub-acute ruminal acidosis may occur (Chen *et al.,* 2012). Thus, an insight into the underlying mechanism that regulate rumen microbial community and fermentation would be desirable. Hence, the present study was conducted to evaluate three different levels of grain in concentrate for its effect on rumen fermentation, microbial profile and *in vitro* digestibility.

MATERIALS AND METHODS

The buffaloes used in this experiment were housed at Animal Nutrition Shed, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India. The shed was well ventilated with separate provision for feeding and watering. All procedures regarding animal handling and treatments within this study were approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, Ministry of Environment and Forest, GOI).

The experiment was carried out on three fistulated adult male Murrah (average body weight 415±10.2 kg) buffaloes in an experiment based on 3×3 switch over design. The animals were fed concentrate (Table1) to meet their requirements (ICAR, 2013) and were divided in three groups, high, moderate and low grain. The proportion of maize grain in ration of high, medium and low grain groups was at 64%, 44% and 23% of DM intake respectively. After feeding for 21 days, the rumen liquor and content (for enzyme) of buffaloes were collected at 0 and 4 h of post-feeding on two consecutive days. The samples were transported to the laboratory in an ice bucket and immediately processed for further sample preparation.

An *in vitro* feed fermentation study was carried

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Table1. Ingredient composition of concentrate fed to buffaloes

Ingredient	High Grain	Medium Grain	Low Grain
	Ingredient composition (g/kg fed basis)		
Wheat bran	-	9	-
Maize	88	75	67
Deoiled soyabean meal	8	11	26
Mineral mixture	2.5	3	4.5
Salt	1.25	1.5	2.5

out utilizing variety of substrates, and the rumen liquor of experimental buffaloes was used as inoculum. The substrate tested were: hays (maize, oat and berseem), roughages (wheat straw, paddy straw and sugarcane bagasse) and mixed diets comprising of different ratios of concentrate mixture and wheat straw (75:25, 50:50 and 25:75). The substrates were dried and ground to pass 1 mm sieve. Exactly weighed substrate (200 mg±10 mg) was transferred in already calibrated syringes of 100 ml capacity in which 30 ml of medium including 10 ml rumen liquor was dispensed anaerobically following standard protocol (Menke and Steingass, 1988). The syringes were incubated for 24 h at 39°C. Each treatment was repeated in triplicate for two consecutive days. After 24 h of incubation, total gas was measured by recording the displacement of piston in the syringe and subtracted from the gas produced in blank syringes to get net gas production. Methane was estimated using gas chromatograph (Nucon-5765) fitted with flame ionization detector (Agarwal *et al.,* 2008). The incubated medium was used for volatile fatty acid (VFA) estimation (Cottyn and Boucque, 1968). Lactic acid was estimated using procedure of Baker and Summerson (1941). After 24 h incubation, the syringe contents were transferred quantitatively by repeated washings with neutral detergent solution in

Table 2. Primers for real time PCR for absolute quantification of rumen microbes

Primer Name	Primer Sequence	Amplicon Size	Annealing Temp	Reference Reference
Bacteria	F-5'CGGCAACGAGCGCAACCC-3'	130	60	Denman, and
	F-5'CCATTGTAGCACGTGTGTAGCC-3'			McSweeney,
Fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC	110	60	2005
	CAAATTCACAAAGGGTAGGATGATT			
Protozoa	316f, 52 -GCTTTCGWTGGTAGTGTATT-32 ;	223	55	Sylvester *et al.,*
	539r, 52 -CTTGCCCTCYAATCGTWCT-32			2004
Methanogen	F 5'-TTC GGT GGA TCD CAR AGR GC-3'R	140	60	Denman, and
	5'-GBA RGT CGW AWC CGT AGA ATC C-3			McSweeney,
				2005
Ruminococcus	F-5'CCCTAAACAGTCTTAGTTCG-3'R-5'	175	60	Kobayashi and
albus	CCTCCTTGCGGTTAGAACA-3'			Koike, 2001
Ruminococcus	F5'CGAACGGAGATAATTTGAGTTTACTTAGG3'	132	60	Tajima *et al.,* 2001
flavefaciens	R-5'CGGTCTCTGTATGTTATGAGGTATTA-3'			
Fibrobacter	F-5'GTTCGGAATTACTGGGCGTAAA-3'			
succinogenes	R-5'CGCCTGCCCCTGAACTATC-3'	121	60	
Butyrivibrio	F-5'TCTGGAAACGGATGGTA-3'			
fibrisolvens	R-5'CCTTTAAGACAGGAGTTTACAA-3'	284	52	

spout less beaker and *in vitro* true digestibility (IVTD) was estimated as per the procedure of Van Soest *et al.* (1991) and expressed as g/kg.

5 g of whole rumen content collected from 3 buffaloes was transferred to 100 ml beakers and mixed with PBS (pH 6.8) at 5 ml/g, carbon tetrachloride (1 ml/g) and 0.4% of lysozyme solution (1 ml/g) as described by Agarwal *et al.* (2000). The protein content of the enzyme samples was estimated following the method of Lowry *et al.* (1951). Enzyme activities were expressed as µmoles of reducing sugars per minute per ml of enzyme samples under assay conditions. Specific activity of enzymes was expressed as IU per mg protein.

Total genomic DNA from the rumen liquor was extracted following the procedure of (Yu and Morrison (2004) by using Qiagen stool kit. For preparation of standard curve, the purified PCR product was cloned using specific primer and the plasmid was serially diluted to make a standard curve after calculating copy number (Ritalahti *et al.,* 2006). 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 mix, 0.6 µl of each primer (10 µM) and nuclease free water (to make up 20 µl of volume). The primer sequences and conditions were as described in table 2. The relative quantification of different microbial groups (total bacteria, total fungi, total protozoa, methanogen, Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes and Butyrivibrio fibrololvens) was done with real-time PCR (CX1000 Touch, BIORAD). The qPCR was standardized for absolute quantification of bacterial cell in per gram of rumen content. 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. The plasmid was serially diluted to make standard curve and the copy number was calculated against respective primers given is Table 2.

Statistical analysis

All data were analyzed as using general linear model of SPSS (SPSS, 2016), with contrast analysis for *in vitro* experiments, to analyze the effect of substrate, inoculum and their interaction. The means were compared using Duncan multiple comparison test. Variability in the data is expressed as the standard error means (SEM) and a probability level of $P<0.05$ was considered to be statistically significant and the P

Table 3. *In vitro* fermentation pattern with different ratio of concentrate and roughage

Substrate(S)		Diet (Grain)			SEM	P Value		
		High	Medium	Low		S	Diet	S*Diet
Gas produced (ml)/g DM								
Hay	Mean	93.38^{b}	106.54^{ab}	116.46^{a}	2.84	0.001	0.007	0.983
Roughage	Mean	75.55^{a}	52.37^{b}	66.43^{ab}	3.34	0.001	0.024	0.875
Mixed diet	Mean	107.11^{c}	128.52^{b}	164.04^{a}	3.06	0.001	0.001	0.944
***in vitro* true digestibility (IVTD)**								
Hay	Mean	52.82^{b}	60.64^{a}	63.12^{a}	0.68	0.011	0.000	0.059
Roughage	Mean	38.66^{b}	45.56^{a}	39.40^{b}	0.99	0.014	0.012	0.269
Mixed diet	Mean	51.97^{b}	58.56^{b}	71.16^{a}	1.23	0.022	0.000	0.591
Methane (ml)/g Digested DM								
Hay	Mean	32.69	31.98	33.97	0.90	0.000	0.661	0.963
Roughage	Mean	38.34^{a}	24.58^{b}	32.39^{ab}	1.46	0.000	0.002	0.918
Mixed diet	Mean	37.41	40.13	41.28	1.21	0.000	0.351	0.574

$^{a-b}$Mean values within a row with unlike superscript letters were significantly different for each dietary treatment ($P<0.05$)

values between 0.05 and 0.10 were considered as a trend. For simplicity of data presentation, mean value for substrates (hay, roughage and mixed diet are presented.

RESULTS AND DISCUSSION

There are currently a number of methane mitigation strategies being investigated to reduce emissions from ruminants, including improved genetics and overall health of animals (potentially leading to reduced herd sizes while maintaining milk yields), use of supplements that reduce methane emissions and immunization against methanogens. However, the most potential strategy is better feed and feeding systems *i.e.* dietary manipulation of the rumen microbiome. In our study, we observed a decrease in methane production in medium grain group (roughage as substrate), which indicates that dietary composition plays major role in deciding methane production. With the information that rumen microbes are very resilient to changes in rumen, manipulation of diet may be used as a successful strategy to decrease methane production in rumen.

In this study, total gas production (ml/g DM) in 24 h increased significantly in low grain diet by 23.7% when compared with high grain treatment whereas medium grain diet is comparable with both diets, (Table 3). Within the three hays, maize showed higher gas production (P<0.01) when compared with other two hays (oat and

Table 4. Microbial profile with different ratio of concentrate and roughage

	Time (h)	Diet (Grain)			Mean	SEM	P value		
		High	Medium	Low			Diet (D)	Time (T)	D*T
Total Bacteria	0	10.09	10.12	10.14	10.12	0.049	0.964	0.665	0.957
	4	10.15	10.18	10.15	10.16				
	Mean	10.12	10.15	10.14					
RA	0	5.70	5.77	5.66	5.71	0.094	0.962	0.476	0.946
	4	5.62	5.55	5.55	5.58				
	Mean	5.66	5.66	5.61					
FS	0	8.68	8.92	9.03	8.87	0.079	0.893	0.803	0.402
	4	8.94	8.79	8.77	8.83				
	Mean	8.81	8.85	8.90					
Methanogen	0	8.08	8.29	7.75	8.04	0.067	0.061	0.710	0.394
	4	8.28	8.08	7.92	8.09				
	Mean	8.18	8.19	7.83					
RF	0	6.52	6.40	6.51	6.48	0.094	0.837	0.661	1.00
	4	6.60	6.48	6.60	6.56				
	Mean	6.56	6.44	6.56					
Total Fungus	0	7.11	7.10	7.18	7.13[a]	0.064	0.827	0.021	0.863
	4	7.52	7.36	7.45	7.44[b]				
	Mean	7.32	7.23	7.31					
Protozoa	0	8.31	8.18	7.70	8.06	0.183	0.504	0.637	0.964
	4	8.41	8.29	8.01	8.24				
	Mean	8.36	8.24	7.85					
BF	0	7.11	7.08	7.08	7.09	0.071	0.822	0.286	0.889
	4	7.33	7.14	7.27	7.24				
	Mean	7.22	7.11	7.17					

[a-b]Mean values within a column with unlike superscript letters were significantly different for each dietary treatment (P<0.05); RA, *Ruminococcus albus*; RF, *R. flavefaciens*, FS, *Fibrobacter succinogenes*; BF, *Butyrivibrio fibrisolvens*

Table 5. *In vitro* metabolites with different ratio of concentrate and roughage

Substrate(S)		Diet (Grain)			SEM	P Value		
		High	Medium	Low		S	Diet	S*Diet
NH_3N (mg/dl)								
Hay	Mean	10.89[a]	6.69[b]	5.72[b]	0.69	0.656	0.009	0.996
Roughage	Mean	12.71[a]	6.11[b]	5.61[b]	0.70	0.999	0.000	0.990
Mixed diet	Mean	12.05[a]	7.25[b]	6.92[b]	0.70	0.833	0.007	1.000
Total VFA (mM/dl)								
Hay	Mean	5.41	5.05	6.10	0.17	0.24	0.53	0.46
Roughage	Mean	5.46	5.00	5.31	0.26	0.49	0.08	0.07
Mixed diet	Mean	5.88	5.71	6.05	0.24	0.23	0.07	0.12

[a-b]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment (P<0.05)

berseem). In roughage substrates, gas production increased (P<0.01) by 30.68% in high grain diet in comparison with medium grain diet and wheat straw shows high gas production (P<0.01) with respect to paddy straw and sugarcane bagasse (Table 3). The IVTD was increased (P<0.01) by 8.27% for berseem hay in comparison with maize and oats hay (Table 3). IVTD of sugarcane begasse was increased (P<0.01) by 15.27% as compared to wheat straw and paddy straw, medium grain diet showed higher IVTD (P<0.01) in comparison to other two diets (20:80 and 80:20 concentrate: roughage). Methane production (ml/g digested DM) was lower by 35.89% in medium grain diet (P<0.01) roughages as compared to high grain diet (Table 3).

Feeding of concentrate ensures optimum maintenance and production in ruminants. However, how the level of concentrate especially grain, affects the rumen fermentation, enzymes and microbes is key factor for optimum production and maintenance of animals. In this study, the decrease in feed degradability observed in our study is in agreement with reports from other studies (Steen *et al.,* 2002; Huuskonen *et al.,* 2007; Keady *et al.,* 2007), which indicate adverse effect of higher level of grain on rumen microbes.

The population density of total bacteria, *F. succinogenes, R. flavefaciens* and *R. albus, methanogens* and fungi was similar (P>0.05) in all the three diets. There was an effect of post-feeding time on some microbes as the population of total fungi was higher (P<0.02) at 4 h post feeding as compared to 0 h feeding (Table 4). The depression in fibre digestibility in the rumen and in the total digestive tract from inclusion of rapidly fermentable carbohydrate, such as concentrate had been reported before (Fahmy *et al.,* 1984; Huhtanen and Jaakkola, 1993) in grass silage based diet, and the depression rate increased sharply after 60% level of concentrate. In contrast to our findings, feeding of different concentrate roughage ratios was found to have no drastic effect on rumen environment (Wanapat and Pimpa, 1999).

The activities of various rumen enzymes like carboxymethylcellulase, xylanase, α-glucosidase, β-glucosidase, amylase and acetyl esterase were affected neither by the diet nor by the post feeding period (Table 6). Ammonia nitrogen production was higher (P<0.05) in high grain fed buffaloes as compared to low grain fed buffaloes (Table 6). Similar observations were made with *in vitro* ammonia nitrogen with all three substrate classes (Table 5). Production of total volatile fatty acids (VFAs) was similar (P>0.05) among treatments in both *in vivo* and *in vitro* experiment.

Higher Ammonia nitrogen and lactic acid concentration observed in the rumen liquor of high grain fed buffaloes in the present study is in agreement with Ogata *et al.*(2019) who reported high lactic acid concentration in cattle fed with high-concentrate diet. In our study the ruminal lactic acid concentrations were higher during high grain diet under both *in vivo* and *in*

Table 6. Specific enzyme activity and certain metabolites (*in vivo*) with different ratio of concentrate and roughage

	Time (H)	Diet(Grain)			Mean	SEM	P value		
		High	Medium	Low			Diet (D)	Time (T)	D*T
CMCase	0	2.13	2.57	1.97	2.22	2.26	0.91	0.87	0.81
	4	2.44	2.19	2.27	2.30				
Mean	2.28	2.38	2.12	2.26					
MCCase	0	1.10	1.13	1.16	1.13	1.02	0.83	0.51	0.89
	4	0.88	0.71	1.15	0.91				
	Mean	0.99	0.92	1.15	1.02				
Amylase	0	3.18	4.98	3.62	3.92	3.79	0.95	0.88	0.85
	4	3.61	3.22	4.12	3.65				
	Mean	3.39	4.10	3.87	3.79				
α-Glucosidase	0	7.48	5.44	5.12	6.01	5.43	0.70	0.67	0.93
	4	6.13	5.63	2.80	4.85				
	Mean	6.80	5.54	3.96	5.43				
β-Glucosidase	0	0.48	0.38	0.28	0.38	0.40	0.44	0.61	0.82
	4	0.50	0.38	0.41	0.43				
	Mean	0.49	0.38	0.34	0.40				
Xylanase	0	6.80	7.18	6.14	6.70	6.59	0.98	0.87	0.83
	4	6.77	5.87	6.80	6.48				
	Mean	6.78	6.52	6.47	6.59				
Protease	0	60.22	66.61	65.92	64.25	69.06	0.85	0.57	0.97
	4	64.61	77.37	79.65	73.88				
	Mean	62.42	71.99	72.78	69.06				
Acetyl esterase	0	5.73	5.36	5.32	5.47	5.12	0.85	0.55	0.77
	4	4.21	5.78	4.32	4.77				
	Mean	4.97	5.57	4.82	5.12				
Urease	0	0.90	1.48	0.81	1.06	1.03	0.66	0.86	0.73
	4	0.77	1.06	1.15	0.99				
	Mean	0.83	1.27	0.98	1.03				
NH_3N(mg/dl)	0	9.72	5.39	4.64	6.58	0.56	0.005	0.758	0.975
	4	9.73	5.83	5.24	6.93				
	Mean	9.72[a]	5.61[b]	4.94[b]					
Lactic Acid	0	1.26	1.28	1.15	1.23	0.03	0.044	0.932	0.333
(mg/dl)	4	1.40	1.21	1.06	1.23				
	Mean	1.33[a]	1.25[ab]	1.11[b]					
TVFA(mM/dl)	0	9.19	7.72	10.64	9.18	0.63	0.184	0.252	0.065
	4	11.15	10.14	11.55	10.94				
	Mean	10.17	8.93	11.10					
A/P	0	3.66	3.75	3.80	3.73	0.12	0.057	0.232	0.059
	4	3.39	3.50	3.34	3.41				
	Mean	3.52	3.63	3.57					

[a-b]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment (P<0.05)

vitro conditions. High ammonia production in rumen with high grain may be due to proliferation of high ammonia producing microbes (HAP), which rapidly ferment concentrate to produce ammonia in rumen. In the present study, the volatile fatty acid profile did not change during *in vivo* and *in vitro* study. Similar to our findings, Hoover *et al.* (2006) reported that the proportion of VFAs (acetate, propionate, and butyrate) was not affected by energy sources. However, Wanapat and Pimpa, (1999) and Oagata *et al.* (2019) reported that VFA in the rumen liquor increased with the increase in the proportion of concentrate mixture in the diet of animals. Animals on the 'hay only' diet showed a more efficient redirection of H_2 into other microbial products in comparison to hay: concentrate diet. A metabolomic study showed that there was an increase in the levels of amino acids, organic and nucleic acids in the liquid phase of the rumen contents in diets, along with decreased methanogenesis, which suggests that there may be enhanced microbial protein synthesis with an alteration in the rumen microbiota, with an increase in the ratio of Bacteroidetes and Firmicutes and a decrease in Archaea and Synergistetes (Martinez-Fernandez *et al.,* 2016). Mathews *et al.* (2019) conducted a study with animals fed either a hay: roughage diet or a hay: concentrate diet in the ratio of 60:40. Additionally an anti-methanogenic compound (chloroform) was also included in the diet. Results showed that with increasing levels of chloroform, there was an increase in H_2 expelled and CH_4 production decreased.

In our study, rumen microbial profile remained unaltered with the three levels of grain, with equally similar observation in rumen enzymes pattern. This may be due to the resilient nature of rumen microbes (and enzymes produced by them) which adapts very rapidly to changing interventions.

CONCLUSIONS

From the present study, it may be concluded that rumen fermentation and its functions are strongly driven by dietary interventions, and thus affects optimum production in animals. Further, a higher grain level is not advised in ruminant animals as it affects the rumen fermentation in unfavorable manner.

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