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Spatial and Temporal Distribution of Microbes and Enzyme Activity in the Rumen of Buffaloes

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ABSTRACT

Information about abundance of rumen microbes is a prerequisite to assess the fermentation in rumen during any dietary intervention. However, conventional techniques are not able to enumerate majority of microbes as majority of rumen microbes are uncultivable. Real time PCR (qPCR) has successfully been used for quantification of various rumen microbes like rumen cellulolytic bacteria, protozoa, fungi, methanogens *etc.* In this experiment, the whole rumen content (WRC) was squeezed to particulate matter (PM) and squeezed rumen liquor (SL), whereas, the fourth fraction, strained rumen liquor (SRL) of rumen content was obtained by filtering through a probe with double layer of muslin cloth. The population of total bacteria, fungi, *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes*, total methanogens, *Butyrivibrio fibrisolvens* and protozoa were estimated in different fractions of rumen content at 0, 4 and 8 h post-feeding by real time PCR using specific primers. The numbers of these microbes were significantly ($P<0.001$) higher in WRC and PM as compared to SRL and RL. The activities of carboxymethylcellulase (CMCase), avicelase, amylase, xylanase, β-glucosidase and urease were significantly ($P<0.05$) higher in WRC and PM as compared to SRL and RL. The activities of CMCase and urease were higher ($P<0.05$) at 4 and 8 h post feeding, whereas, rest of the enzymes were not affected. There was no effect of time of sampling on the population of rumen microbes explored in this experiment. It appeared that WRC or PM fraction provided true picture of microbial and enzyme profiles responsible for fibre degradation in the rumen. The increase in enzyme activity at a particular time of sampling was not associated with population size of the specific microbes or specific activity of enzymes.

Key words: Buffalo, Enzymes, Microbes, Rumen content fractions, Real time PCR

INTRODUCTION

Rumen microbes have a mutualistic relationship with their hosts, and symbiotic and/or antagonistic relationship among themselves (Kamra, 2005). The rumen microbes, predominantly bacteria, fungi and protozoa, secrete an array of fibrolytic enzymes (Chen *et al.,* 2008) including micro-crystalline cellulase, caboxymethylcellulase, xylanase *etc.* and this complex pool of enzymes are responsible for degradation of plant based fibrous feeds. The major culturable fibre degrading bacteria are represented by *F. succinogenes, R. flavefaciens, R. albus* and *B. fibrisolvens* based on conventional cultivation techniques and their contribution to fibre degradation is much larger than the other microbes (Wanapat and Cherdthong, 2009; Dai *et al.,* 2015). *B. fibrisolvens* and *Prevotella ruminicola* are other major fibre degrading bacteria which contribute to carbohydrate and fibre utilization (Flint *et al.,* 2008). For fibre degradation, the microbial population attached to feed particles is the most important as about 80-90% of carboxymethyl cellulase, avicelase and xylanase are from the microbes attached with the feed particles or particulate matter (Agarwal *et al.,* 2000). But the microbes identified so far as the main fibre degrading microbes are the cultivable microbes, whereas, there are other numerous bacteria which are functional but uncultivable under laboratory condititon (Kobayashi, 2006). Real time PCR has successfully been used for quantification of various rumen microbes like rumen cellulolytic bacteria, protozoa, fungi, methanogens etc (Tajima *et al.,* 2001; Koike and Kobayashi, 2001).

The present study has been undertaken to establish the distribution of various microbes by using real-time PCR and the major microbes and fibre degrading enzymes secreted by these microbes in the

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rumen and their changing pattern with the type of sample and feeding time. Some *in vitro* studies reported change in rumen microbial count for all major microbes after 24 h of incubation in Rusitec (Lengowski *et al.,* 2016), whereas, there are also reports of no effect of period of sampling on rumen microbes and enzyme activity (Li *et al.,* 2009; Kala *et al.,* 2017, 2020).Most of the studies looking into rumen microbes attached to different rumen content fractions have used high throughput sequencing. de Mulder *et al.* (2017) has reported high abundance of celllulolytic and the associated secondary bacteria in solid phase. Bowen *et al.* (2018) also reported high abundance of methanogens on Holstein Friesian (HF) cattle grazing on white clover *vs.* perennial ryegrass, whereas de Mulder *et al.* (2017) reported similar abundance in solid and liquid fraction of rumen. The change in pattern of rumen microbes and enzymes produced by them affect the extent of fibre degradation in rumen. The studies available have explored the temporal variation in microbes using *in vitro* methods, and availability of studies using *in vivo* models is very meagre. This study explores the shift in population of rumen microbes and the enzymes produced by them at different periods and fractions.

MATERIALS AND METHODS

The experiment was carried out on three fistulated male buffaloes fed control diet of wheat straw and concentrate mixture (wheat bran 37%, maize 41%, de-oiled soybean meal 19%, mineral mixture 2% and common salt 1%) in equal proportion to meet nutrient requirements for maintenance (ICAR, 2013). The whole rumen content for enzyme estimation and microbial enumeration was collected at 0 (just before feeding), 4 and 8 h post-feeding on two consecutive days, after 21 days of feeding. The samples were transported to the laboratory in an ice bucket and processed immediately. The rumen content was separated into four fractions i.e. the whole rumen content (WRC), and it was squeezed to have particulate material (PM, the solid fraction) and the squeezed liquid (SL) portion. The fourth fraction was SRL (strained rumen liquor) obtained by filtering through two layers of muslin cloth.

For enzyme extraction, either 5 g particulate material, whole rumen content, SL or SRL were mixed

Table 1. PCR primers for real time PCR

Primer Name	Primer Sequence	Amplicon (bp)	Annealing temp(°C)	Reference
Bacteria	F-5'CGGCAACGAGCGCAACCC-3' R-5'CCATTGTAGCACGTGTGTAGCC-3'	130	60	Denman *et al.,* 2006
Fungi	F-GAGGAAGTAAAAGTCGTAACAAGGTTTC R-CAAATTCACAAAGGGTAGGATGATT	110	60	
Methanogen	F 5'-TTCGGTGGATCDCARAGRGC-3'R R-5'-GBARGTCGWAWCCGTAGAATC C-3	140	60	
Ruminococcus flavefaciens	F5'CGAACGGAGATAATTTGAGTTTACTTAGG3' R-5'CGGTCTCTGTATGTTATGAGGTATTA-3'	132	60	
Fibrobacter succinogenes	F-5'GTTCGGAATTACTGGGCGTAAA-3' R-5'CGCCTGCCCCTGAACTATC-3'	121	60	
Ruminococcus albus	F-5'CCCTAAACAGTCTTAGTTCG-3' R-5'CCTCCTTGCGGTTAGAACA-3'	175	60	Koike and Kobayashi, 2001
Protozoa	F-316f, 5'-GCTTTCGWTGGTAGTGTATT-3' R-539r, 5'-CTTGCCCTCYAATCGTWCT-3'	223	55	Sylvester *et al.,* 2004
Butyrivibrio fibrisolvens	F-5'TCTGGAAACGGATGGTA-3' R-5'CCTTTAAGACAGGAGTTTACAA-3'	284	60	Chen and Weimer, 2001

with phosphate buffer (0.1M, pH 6.8), lysozyme (0.4%) solution and carbon tetrachloride and incubated at 39°C for 3 h and the clear supernatant obtained after centrifugation was used as a source of microbial enzymes (Hirstov *et al.,* 1999).

For measuring the activities of carboxymethyl cellulase and xylanase, the reaction mixture contained 1 ml phosphate buffer (0.1 M pH 6.8); 0.5 ml enzyme and 0.5 ml of either carboxymethylcellulose (1.0%) or xylan (0.25%). The reaction mixture for α-amylase contained 0.5 ml buffer, 0.25 ml starch (1%) and 0.25 ml enzyme. The reaction mixtures were incubated for 60, 15 and 30 min, respectively, at 39°C and the reducing sugars released were estimated (Miller, 1959). β-glucosidase activities were estimated using p-nitrophenyl- α-glucopyranoside and p-nitrophenyl-α-glucopyranoside as substrate, respectively (Shewale and Sadana, 1978). Urease activity was measured by incubating the enzyme sample (0.25 ml) with urea (0.25 ml of 0.1 M) for 15 min and released ammonia nitrogen was estimated (Weatherburn, 1967). Protease activity was measured using azocasein (Sigma, St. Louis, USA) as a substrate (Brock *et al.,* 1982) and the enzyme unit was expressed as µg protein hydrolysed/min/ml.

Total genomic DNA was extracted as per procedure described by Yu and Morrison (2004) which included bead beating of samples mixed with lysis buffer followed by purification of DNA by using a kit (A9281, Wizard SV Gel and PCR clean up system). The quality and quantity of the DNA was checked by electrophoresis (0.8% agarose) and nanodrop qPCR was standardized for absolute quantification of microbial cell per gram of rumen content/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* by using Transform Aid™ Bacterial Transformation Kit (Fermentas). The plasmid with insert was extracted and copy number was calculated (Ritalahti *et al.,* 2006). The plasmid was serially diluted to make standard curve and the copy number of unknown sample was calculated. 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. The cycling conditions consisted of initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and specific annealing temperature for 15 sec. The specific primers used for qPCR with their annealing temperature and product size are given in Table 1.

RESULTS AND DISCUSSION

The results of microbial profile including total bacteria, *Ruminococcus flavefaciens*, *R. albus*, methanogens, protozoa and *Fibrobacter succinogenes* in the rumen of buffalo (Table 2) studied with real time PCR indicated that the number of rumen microbes was higher ($P<0.05$) in WRC and PM as compared to those in SRL and SL. However, there was no difference in the temporal distribution (at 0, 4 and 8 h post-feeding) of microbial population. This clearly indicated that majority of the rumen microbes, especially fibre degraders are attached with particulate material/solid portion of rumen content. Relative microbial diversity was studied to see how the rumen microbial profile changes in other fractions with respect to whole rumen content. It was calculated by taking the absolute count of microbes in whole rumen content as 1, and microbial count for other sample type was expressed in relation to whole rumen content (Table 3). The relative count

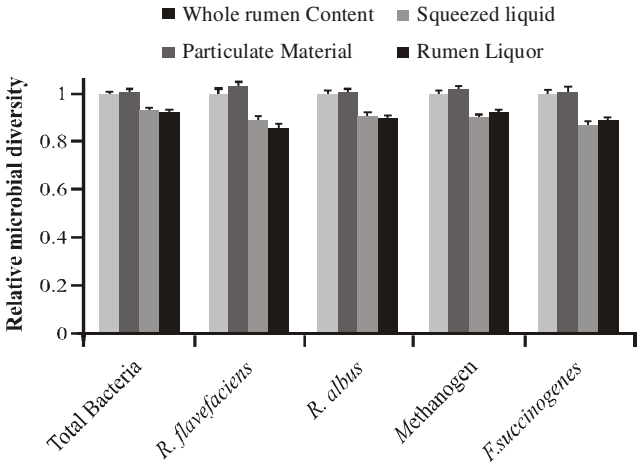

Fig. 1. Relative microbial diversity of major rumen microbes in various fractions of rumen content of buffaloes

also exhibited similar trend as absolute count of microbes (Fig. 1). It clearly indicated that microbial count in PM is generally similar, sometimes even numerically higher than WRC. The relative count in other two fractions, namely SL and SRL were observed to have similar microbial count.

The information on microbial diversity of buffalo rumen available till date is primarily based on conventional techniques of microbial cultivation and the information available is scanty as compared to other domesticated (cattle, sheep and goat) and wild ruminants (Kamra and Pathak,1996). This scanty knowledge has limited our understanding of the environment and requirements of target microbes in buffalo rumen as majority of the rumen bacteria are uncultivable under lab condititon. However, real time quantification of bacteria in the rumen has made it possible to quantify the absolute number of some of these

Table 2. Distribution of microbes (Log_{10}) in different fractions of rumen contents at different hrs of post-feeding

Primers	Time(h)	WRC	PM	SL	SRL	Mean	SEM	P value		
								T	F	T*F
Bacteria	0	11.41	11.50	10.64	10.57	11.03	0.067	0.970	<0.001	0.806
	4	11.64	11.28	10.39	10.83	11.03				
	8	11.38	11.48	10.33	10.79	11.00				
	Mean	11.4[a]	11.42[a]	10.45[b]	10.73[b]					
F. succinogenes	0	10.59	10.66	9.18	9.40	9.96	0.071	0.188	<0.001	0.785
	4	10.82	10.32	8.96	8.93	9.76				
	8	10.42	10.40	8.66	9.07	9.64				
	Mean	10.6[a]	10.46[a]	8.93[b]	9.13 [b]					
R. albus	0	7.78	7.85	7.07	6.96	7.41	0.047	0.165	<0.001	0.190
	4	7.92	7.52	6.49	7.07[ab]	7.25				
	8	7.69	7.76	6.41	6.93	7.20				
	Mean	7.80[a]	7.71[a]	6.66[b]	6.99[b]					
R. flavefaciens	0	8.47	8.72	7.52	7.24	7.99	0.060	0.928	<0.000	0.618
	4	8.77	8.48	7.17	7.42	7.96				
	8	8.57	8.73	7.23	7.55	8.02				
	Mean	8.60[a]	8.64[a]	7.31[b]	7.40[b]					
B. fibrisolvens	0	9.48	9.58	8.44	8.21	8.93	0.086	0.662	<0.001	0.548
	4	9.69	9.42	7.64	8.20	8.74				
	8	9.40	9.56	7.73	8.53	8.80				
	Mean	9.52[a]	9.52[a]	7.93[b]	8.31[b]					
Methanogens	0	8.60	8.75	7.75	7.94	8.26	0.068	0.247	<0.001	0.795
	4	8.95	8.56	7.55	7.81	8.22				
	8	8.53	8.59	7.18	7.70	8.00				
	Mean	8.69[a]	8.63[a]	7.49[b]	7.82[b]					
Protozoa	0	9.85	9.82	9.32	9.62	9.65	0.105	0.054	0.034	0.946
	4	10.14	9.60	8.94	9.34	9.50				
	8	9.45	9.01	8.44	9.22	9.03				
	Mean	9.81[a]	9.48[ab]	8.90[b]	9.39[ab]					

*WRC, whole rumen content; PM, particulate matter; SL, squeezed liquid; SRL, strained rumen liquor; T, time; F, fraction

Table 3. Relative count of microbes in different fractions of rumen contents at different periods after feeding

Fraction	WRC	PM	SL	SRL	Mean	SEM	P value
Bacteria	1[a]	1.01[a]	0.93[b]	0.93[b]	0.97	0.010	<0.001
R. albus	1[a]	1.01[a]	0.91[b]	0.90[b]	0.95	0.015	<0.001
R. flavefaciens	1[a]	1.03[a]	0.89[b]	0.86[b]	0.94	0.021	<0.001
Methanogens	1[a]	1.02[a]	0.90[b]	0.92[b]	0.96	0.015	<0.001
Protozoa	1[a]	1.00[a]	0.95[b]	0.98[b]	0.98	0.008	0.026
F.succinogenes	1[a]	1.01[a]	0.87[b]	0.89[b]	0.94	0.017	<0.001

*WRC, whole rumen content; PM, particulate matter; SL, squeezed liquid; SRL, strained rumen liquor; T, time; F, fraction

rumen bacteria (Malmuthuge *et al.,* 2012). In corroboration with this study, Li, *et al* (2009) reported no effect of sampling time (at three time points: 3h before feeding, 3h after feeding and 9h after feeding) or location in rumen from where samples were collected (cranial dorsal, cranial ventral, central rumen, caudal

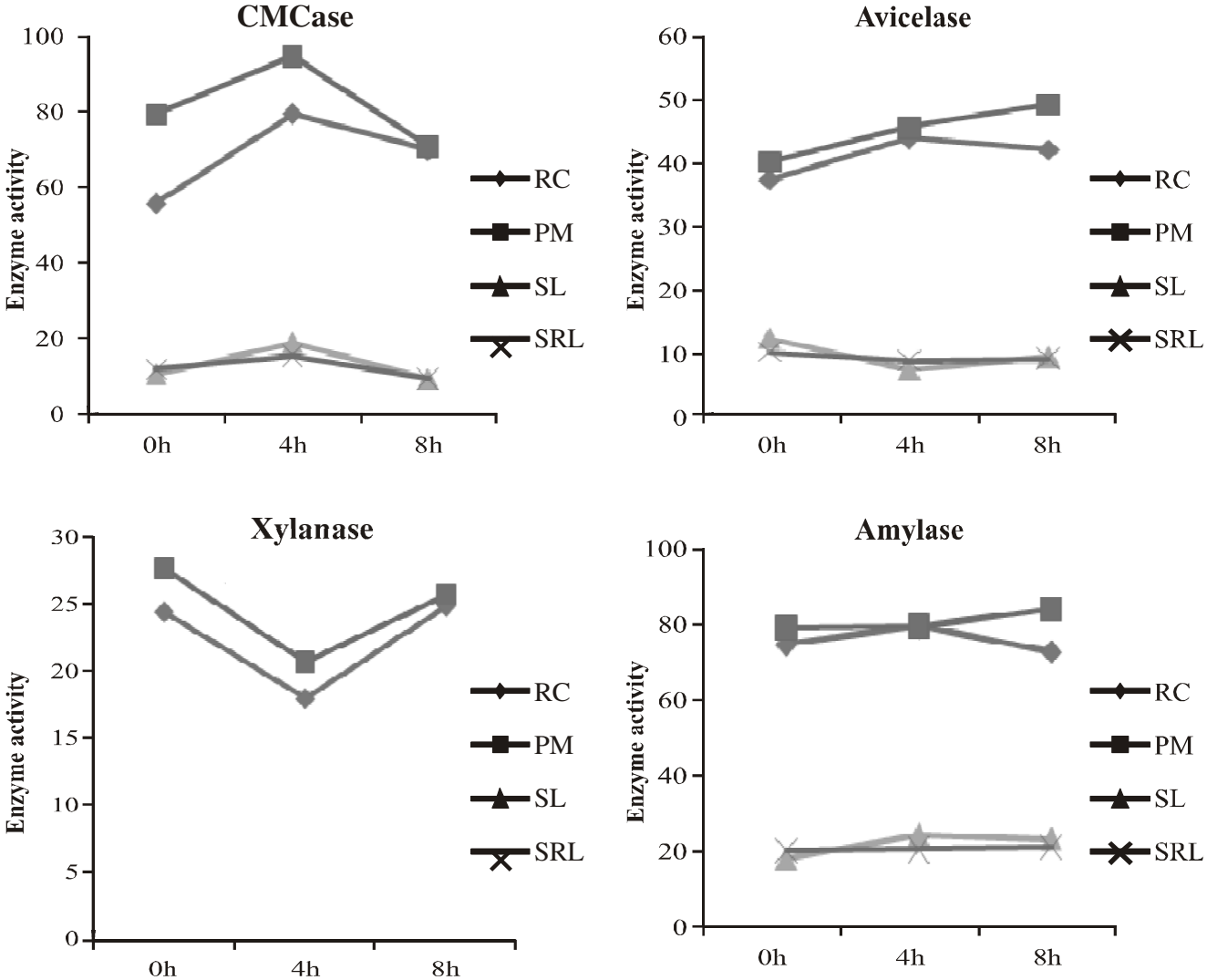

Fig. 2. Temporal variation in major fibrolytic enzymes in buffalo rumen

Table 4. Enzyme activities (µmol/ml/min/100g RC/RL) in different fractions of rumen contents at different post-feeding periods

Enzyme	Time (Hr)	RC	PM	SL	SRL	Mean	SEM	P value		
								T	F	T*F
CMCase	0	55.93	79.63	10.68	11.88	39.53 [b]	1.94	0.032	<0.001	0.552
	4	79.59	94.93	19.07	15.54	52.28[a]				
	8	70.04	71.00	9.35	9.35	39.93[ab]				
	Mean	68.52[a]	81.85 [a]	13.03[b]	12.26[b]					
MCCase	0	37.72	40.56	12.59	10.33	25.30	0.66	0.342	<0.001	0.129
	4	44.30	46.02	7.53	9.04	26.72				
	8	42.54	49.65	9.62	9.33	27.78				
	Mean	41.52[a]	45.41 [a]	9.91[b]	9.57[b]					
Amylase	0	75.41	79.93	18.14	20.42	48.47	5.88	0.977	<0.011	1.000
	4	80.15	80.45	24.59	20.61	51.45				
	8	73.41[a]	85.12[a]	23.45[b]	21.14[b]	50.78				
	Mean	76.32	81.83	22.06	20.72					
Xylanase	0	24.44	27.65	5.47	3.57	15.28	0.82	0.232	0.003	0.699
	4	17.96	20.72	4.14	4.54	11.84				
	8	24.89	25.69	3.90	3.93	14.60				
	Mean	22.43[a]	24.68 [a]	4.50[b]	4.01[b]					
β-glucosidase	0	11.35	10.50	2.87	1.89	6.65	1.01	0.942	0.018	1.000
	4	9.76	9.15	2.79	2.94	6.16				
	8	11.00	11.25	3.07	2.76	7.02				
	Mean	10.70[a]	10.30 [a]	2.91[b]	2.53[b]					
Urease	0	5.94	6.80	1.07	0.71	3.63 [b]	0.21	0.001	<0.001	0.023
	4	5.43	5.86	1.97	2.49	3.93 [b]				
	8	9.38	10.76	2.00	1.68	5.95[a]				
	Mean	6.91 [a]	7.80 [a]	1.68[b]	1.63[b]					
Protease	0	51.34	62.67	10.88	17.25	35.53	4.25	0.945	0.002	0.998
	4	61.33	62.00	15.63	10.63	37.40				
	8	51.33	57.67	15.63	10.88	33.87				
	Mean	54.67[a]	60.78[a]	14.04[b]	12.92[b]					

CMCase, amylase and xylanase: nmol glucose or xylose ml^{-1} min^{-1}; α-glucosidase, β-glucosidase and acetyl esterase, nmol p-nitrophenol ml^{-1} min^{-1}; protease, µg of protein hydrolysed ml^{-1} min^{-1}

dorsal and caudal ventral locations) on rumen microbe population of Holstein cows. Similarly, Kala *et al.,* (2017, 2020) had reported no change in rumen microbial profile and rumen enzyme activity at 0 and 4h post feeding in buffaloes fed various levels of TDN. Lengowski *et al.* (2016) has reported no change during initial periods after adding silage as substrate in Rusitec, but a decrease was observed for all major microbes after 24 h of incubation. In our study it was observed that, the number of these bacteria drastically reduced when liquid portion of rumen is used for analysis. Thus, the whole rumen content or particulate matter seems to be better representative for microbial profiling study. However, the time of sampling was not found to be a factor affecting microbial population.

The polymeric carbohydrate-based crop residues are degraded by a combined activity of a microbiome consisting primarily of bacteria accompanied with protozoa, fungi, archaea and bacteriophages (Klieve and Bauchop, 1988; Kamra, 2005). In order to have better

access to the fibre, the microbes excreting these hydrolytic enzymes must get attached with the substrate. This is even observed in the present experiment that the microbes and enzymes excreted by these microbes were in close association with the particulate material of the partially degraded feed ingredients in the rumen. The enzyme activity also followed similar trend as microbial profile and observed higher activity associated with WRC and PM (Table 4). Activity of CMCase and urease was higher at 4 h and at 8 h post-feeding, whereas no temporal changes in activities of other enzymes were observed. Higher activity of CMCase reflects increased fibre utilization at 4 h post-feeding. Results further indicated that the rumen content and particulate material had higher population of rumen microbes and higher quantity of enzymes (Fig. 2), than the liquid portion from the squeezed and strained rumen liquor. From the results it appears that when enzyme and microbial profiles are to be studied in the rumen content, the whole rumen content should be sampled as majority of the enzymes and microbes are attached with the partially degraded feed particles. The present study revealed that temporal changes appear to have little or no effect on activity of enzymes in rumen.

CONCLUSIONS

The study clearly revealed that the whole rumen content or particulate material seems to be containing majority of rumen microbes and thus the hydrolytic enzymes produced by these microbes. The time of sampling does not appear to affect the microbial number or enzyme activity. Thus, for rumen fermentation studies whole rumen content should be the preferred sample. Due to the lack of existing information and unclutivablity of majority of rumen microbes' culture independent techniques seem to be better approach. These primer based approaches likes real time PCR are better than cultivation based methods but, high throughput techniques involving metagenomics and metatranscriptomics are the holistic approach as they sequence all the DNA/RNA present in a sample. Nonetheless, qPCR provides great deal of information about the microbial profile of rumen microbes.

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