Original Research

Postprandial Changes in Rumen Microflora and Fermentation Pattern in Sheep Fed Paddy Straw Based Complete Feed Supplemented with Probiotics Mix

G. G. Sheikh, Danish Masood, Shakil A. Bhat[1*], A. M. Ganai, Yasir Afzal and Shabir Mir[2]

Division of Animal Nutrition, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-Kashmir, Srinagar-190006, Jammu and Kashmir, INDIA

[1]Division of Animal Biotechnology
[2]Division of Animal Genetics and Breeding

***Corresponding author:** shakilvet@gmail.com

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Abstract

A study was undertaken to evaluate effect of feeding probiotics mix (Saccharomyces cerevisiae 2×10^{10}cfu/g + Lactobacillus acidophilus 6×10^{9}cfu/g) in equal ratio in paddy straw based complete feed @ 3 % of DM, as per the in vitro studies carried to arrive at optimum level of incorporation, on rumen microbial count and fermentation parameters, while complete feed without probiotics served as control. The mean values of total bacterial, fibrolytic bacteria (Fibrobacter succinogenes), fungi and protozoa count showed significantly (P<0.01) higher value at 0h feeding (before feeding) and lowest value at 2 h post feeding with increasing trend thereafter upto 8 h post feeding, irrespective of treatment. Rumen microbes showed significantly (P<0.01) higher counts for probiotics mix supplemented groups than in un-supplemented group. However, regarding methanogens nonsignificant difference was observed between probiotics mix supplemented groups than control.

Key words: Fermentation Parameters, Lambs, Postprandial, Probiotics Mix, Rumen microbes

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Introduction

Concept behind feeding probiotics to livestock is based primarily on potentially beneficial postruminal effects, including improved establishment of beneficial gut microflora (Fuller, 1999). Among probiotics, *Saccharomyces cerevisiae* (brewers and baker's yeast) and *Lactobacillus acidophilus* (lactic acid producing bacteria) has got maximum attention among nutritionists throughout world. These multi-species probiotic preparations have synergistic effects on animal health and performance because of protocooperation and is explained by exchange of certain growth factors such as amino acids, peptides, formate and CO_2

(Timmerman *et al.,* 2005). In ruminants probiotics has shown to improve the rumen-predominant microorganisms (Jouany*et al.,* 1998) and ruminal performance (Kritas *et al.,* 2006), as yeast cultures provides various growth factors, provitamins and micronutrients that stimulate the growth of the bacteria in the rumen (Beharka *et al.,* 1991, Newbold *et al.,* 1995, Wiedmeier *et al.,* 1987) and probiotics consisting lactic-acid-producing bacteria promote the stability of the rumen flora (Beauchemin *et al.,* 2003,Chiquette *et al.,* 2012, Ghorbani *et al.,* 2002).There has been an improvement in average daily gain, feed efficiency, digestibility and rumen fermentation in lambs and kids supplemented with microbial feed additives containing *S. cerevisiae* and *Lactobacillus* (Soren *et al.,* 2012; Whitley *et al.,* 2009; Doto *et al.,* 2011).

There is continuous changes in rumen microbial community both at structural and population levels with time indifferent ways, with different amplitudes and reaching maxima and minima at different times. However, the dynamics of the microbial community within the rumen and how the microbial structure and population change in response to various factors, are not well characterized. There is little information available for the differences in microflora diversity within the rumen and effect of probiotics feeding on rumen microflora. To test this hypothesis, we assessed by quantitative PCR (qPCR) the postprandial changes of rumen microbes in of sheep fed probiotic mix (*Sachromyces cervicea* + *Lactobacillus acidophillus*) supplemented diet. The present study reports, for the first time in India monitoring of different species of microflora in the rumen of sheep by real-time PCR measured by quantifying the rrs gene of each species, in sheep fed paddy straw based complete feed.

Materials and Methods

Animal Handling and Sampling

A feeding trail of 90 days was carried on ten male Corriedale lambs (3-4 months old, 9.25-11.00 kg) of uniform conformation procured from Mountain Research Centre for Sheep and Goat (MRCSG), Faculty of Veterinary Sciences and Animal husbandry, Shuhama, SKUAST-Kashmir. A complete feed was prepared containing paddy straw 50 parts and concentrate mixture 50 parts on DM basis to meet the nutrient requirement of animals as per ICAR (2013). The parts of concentrate mixture were maize 6.0, wheat bran 7.6, deoiled rice bran 9.0, mustard oil cake 5.0, soyabean 20.4, molasses 0.8, mineral mixture 0.8 and salt 0.4. Probiotic mix (*Saccharomyces cerevisiae* 2×10^{10}cfu/g + *Lactobacillus acidophilus* 6×10^{9}cfu/g) in equal ratio was incorporated in complete feed @ 3 % of DM, as per the *in-vitro* studies carried to arrive at optimum level of incorporation of probiotic mix to paddy straw based complete feed for efficient utilization in ruminant system, while complete feed without probiotics served as control. The animals were given measured quantity of experimental feed and *ad lib* water every morning. Animals were housed in well ventilated, hygienic and protected sheds and were allowed to acclimatize for a period of 15 days prior to experimental feeding.

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Microbiological Analyses

At the end of 90 days trail rumen liquor samples were collected from the experimental lambs at 0, 2, 4, 8, and 12 h post feeding to assess the effect of supplementation of probiotics mix in paddy straw based complete feed on the rumen microbial count. One half of the sample was used immediately after withdrawal for the measurement of microbial viable counts, the other half was stored without any preservative at -20^{o}C by aliquots of 100 g for enzymatic analyses.

Genomic DNA Isolation from Rumen Fluid

Total DNA was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA). The yield of total extracted DNA (µg of DNA/g of DM o rumen content) was expressed as the mean of 2 extractions per animal. The total DNA mixture was used as a template in PCR to amplify 16S rDNA. The DNA quantity and quality where checked by 0.8% (wt/v) agarose gel electrophoresis and NanoDrop spectrophotometer (ND 1000, NanoDrop technologies, Inc., Wilmington, DE, USA) at 260 nm.

Species specific PCR primers were used for amplification of target region (target DNA) of the 16SrRNAfor total bacteria and fibrolytic bacteria, ITS1 region for fungi, *mcrA*for methanogens*and* 18SrRNA for protozoa were chosen from the literatures (Table 1).

Table 1: Primers for real time PCR assay

Microbe	Primer sets	Targeted gene	Reference
Total Bacteria	F: 5'-CGG CAACGAGCGCAACCC-3',	16S rRNA	Denman and McSweeney, 2006
	R:5'-CCATTGTAGCACGTGTGTAGCC-3'		
Fibrobacte rsuccinogenes	F: 5'-GTTCGGAATTACTGGGCGTAAA-3'	16S rRNA	Tajima *et al.*, 2001
	R: 5'-CGCCTGCCCCTGAACTATC-3'		
Methanogen	F: 5'-TTCGGTGGATCDCARAGRGC-3',	*mcrA*	*Denmen at al.*, 2007
	R: 5'-GBARGTCGWAWCCGTAGAATCC-3'		
Fungi	F:5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3'	ITS1 region	Denman and McSweeney, 2006
	R: 5'-CAAATTCACAAAGGGTAGGATGATT-3'		
Ciliate Protozoa	F: 5'-GCTTTCGWTGGTAGTGTATT-3'	18S rRNA	Sylvester *et al.*, 2004
	R: 5'-CTTGCCCTCYAATCGTWCT-3'		

All quantification real-time PCR amplification and detection was done using ABI 7500 system software (ABI 7500, USA). The reaction was conducted in a final volume of 20 µl in duplicate in each well for each sample in 96 well PCR plate containing 10.0 µlSyber green mix, 0.6 µl forward primer, 0.6 µl reverse primer, 2.0 µl template and 6.8µl nuclease free water. The plate was sealed and placed in real time thermal cycler (Stratgene MX 3000P thermo cycler). The assay was conducted with the following cycle conditions: one cycle at 50°C for 2 min. and at 95°C for 2 min. for initial denaturation; 40 cycles at 95°C for 15 s and at 60°C for 1 min. for primer annealing and product elongation. The dissociation curve analysis of PCR

end products was performed with 71 cycles at 95°C for 1 min., followed by 60°C for 10 s. A negative blank (without the DNA template) was also run for each primer pair. The 10 fold dilution series of standard plasmid for the respective target was run along with the samples. Amplification of each sample was performed in duplicate. The copy numbers of 16S rRNA genes of all targeted per ml rumen fluid were calculated using equation: (QM× C × DV)/(S × V), where QM was quantitative mean of the copy number, C was DNA concentration of each sample, DV was the dilution volume of the extracted DNA, S was the amount (ng) subjected to analysis and V is the rumen fluid volume subjected to DNA extraction. A linear regressions [r^2 = 0.99 and slope (-3.2 to -4)] were obtained between threshold cycle and quantities of standard for all targets and data generated from the reaction were used for further analysis. Statistical analysis of data was performed by using software of the SPSS, version 20.0, Chicago, USA. The differences were determined by the method of least significant differences at the 5% level ($p< 0.05$) of data in rumen fluid at 0, 2, 4, 6, 8 and 12 h after feeding.

Result and Discussion

The dry matter intake recorded in terms of g/d, % kg body weight (BW) and g/kg$W^{0.75}$ were found to be significantly (P<0.01) higher in probiotic mix supplemented group in comparison to control (Table 2). Our results fall in line with the observations of Garg *et al.* (2009), Hillal *et al.* (2011) and Latif *et al.* (2014).

Table 2: Chemical composition of experimental feeds and feed ingredients

Particulars	Unsupplemented Group	Probiotic Mix Supplemented Group
Ingredients Proportion (%)		
Paddy straw	50	50
Concentrate mix	50	50
Chemical Composition (% DM)		
CP	15.51	15.75
EE	3.15	3.18
CF	21.64	21.64
NFE	49.06	48.8
TA	8.64	8.66
AIA	3.31	3.33
NDF	68.03	67.79
ADF	42.15	42.11
HC	25.88	25.68
Cellulose	34.37	34.41
ADL	5.49	5.15
Ca	1.93	1.94
P	0.59	0.61
Dry Matter Intake		
DMI (g/d) **	575.63±14.37[a]	634.05±15.72[b]
DMI (%kg BW)	4.32±0.02	4.40±0.04
DMI (g/kg $W^{0.75}$) **	82.38±0.64[a]	85.49±0.48[b]

Means superscripted with different letters in column ([ABCD]) for a particular data differ significantly from each other
*** (P<0.01)*

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Rumen Microflora

For quantification of rumen microflora absolute quantification by real-time PCR of the rrs gene originating from each microbial species was done using the previously published primers that were shown to be species-specific (Table 1). The mean values for total bacterial count (Log_{10}) per ml recorded at different hours have been presented in (Table 2). There was significant effect of feeding probiotic mix on total bacterial and fibrolytic bacterial at different time intervals post feeding. Similar results have been reported by (Martin and Michalet-Doreau, 1995; Michalet-Doreau *et al.*, 2002; Bhanderi *et al.*, 2016). Immediately following feeding, the concentration of total bacteria and fibrolytic bacteria (*F. succinogenes*) count decreased and continued to decrease for the first 2-4 hr. However, the proportion of these organisms started to increase immediately and increased to a maximum of some equal to the initial rate at about 12hr after feeding. This decrease in bacterial count postprandial have been attributed to the dilution of ruminal contents by ingested feed (Saro *et al.*, 2015). Similar observations were reported by Lascano *et al.* (2009) who found yeast addition increased number of viable bacteria cells in ruminants with bacterial counts decreased for the first 2 hr after feeding then increased 4 hr post feeding. Saro *et al.* (2015) also reported that total bacterial DNA concentrations decreased at 4 hr after feeding and then increased at 8 h after feeding to values similar ($P>0.05$) to those before feeding. Chaucheyras *et al.* (2010) reported that the supplementation of yeast additive promoted colonization of fibrous substrates by cellulolytic bacteria (*F. succinogenes, R. flavefaciens, B. fibrisolvens*) and fungi but that the degree of stimulation was depending on the nature of the substrate, and on the microbial species targeted. A two- to four-fold increase in the number of 16S rRNA gene copies of *R. albus* and *R. flavefaciens* was also measured with real-time PCR in rumen contents of sheep receiving a high-concentrate diet and yeast (Mosoni *et al.*, 2007).

Regarding methanogens a non-significant effect of probiotic mix supplementation were observed, however time of sampling showed significant effect on population density across period, the counts were found to be highest at 0 hr sampling i.e. before feeding and being least at 2 hr post feeding following the same trend as that of total bacteria and fibrolytic bacteria (Table 3). A 20% decrease in methane production after a 48hr of incubation of mixed rumen microorganisms in the presence of alfalfa and a live yeast product (Lynch and Martin, 2002). Ruminal fungi and protozoa were significantlyhigher at 0 hr sampling and being lowest at 2 hr post feeding (Table 3) probably due to dilution effect caused by intake of feed and water by the animals. The other probable reason may be the pH of the rumen liquor which was found to be lowest at this hour post feeding, since protozoal population is very sensitive to change in pH and may be inhibited or eliminated at low pH (Hungate, 1966). Saro *et al.* (2015) reported relative abundance of fungal DNA values at 4 hr after feeding on solid rumen contents, compared with those at 0 and 8 hr for both diets, which is in agreement with the less relative abundance of fungal DNA observed in our study at 2 hr after feeding in rumen fluid. Saro *et al.* (2015) and Santra *et al.* (1998) reported that protozoa numbers in the liquid phase

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of the rumen decreased after feeding, and this decrease was attributed to the migration of protozoa to colonize feed particles.

Table 3: Average Log_{10} values of rumen microbes at different time intervals in treatment groups

Time Interval (Hours)	Treatment Groups	
	Unsupplemented Group	Probiotic Mix Supplemented Group
Total Bacteria		
0	10.09 ± 0.03^{E}	10.99 ± 0.05^{C}
2	9.69 ± 0.02^{A}	10.24 ± 0.08^{A}
4	9.81 ± 0.02^{B}	10.46 ± 0.10^{AB}
8	9.92 ± 0.02^{C}	10.53 ± 0.11^{B}
12	10.00 ± 0.02^{D}	10.82 ± 0.07^{C}
Fibrobacter succinogenes		
0	9.28 ± 0.01^{C}	9.59 ± 0.10^{B}
2	8.77 ± 0.03^{A}	9.14 ± 0.10^{A}
4	8.85 ± 0.03^{AB}	9.26 ± 0.06^{A}
8	8.91 ± 0.04^{B}	9.38 ± 0.06^{AB}
Methanogens		
0	7.42 ± 0.05^{D}	7.52 ± 0.03^{D}
2	7.09 ± 0.01^{A}	7.12 ± 0.02^{A}
4	7.15 ± 0.01^{AB}	7.17 ± 0.02^{A}
8	7.25 ± 0.03^{BC}	7.29 ± 0.02^{B}
12	7.32 ± 0.05^{CD}	7.41 ± 0.03^{C}
Total Fungi		
0	6.33 ± 0.01^{aC}	6.81 ± 0.02^{cE}
2	6.13 ± 0.01^{bA}	6.15 ± 0.02^{bA}
4	6.16 ± 0.02^{aAB}	6.31 ± 0.03^{bB}
8	6.20 ± 0.03^{aB}	6.41 ± 0.04^{bC}
12	6.30 ± 0.01^{aC}	6.55 ± 0.03^{bD}
Total Protozoa		
0	8.46 ± 0.05^{C}	8.46 ± 0.04^{BC}
2	8.22 ± 0.01^{aA}	8.34 ± 0.01^{bA}
4	8.32 ± 0.01^{aB}	8.40 ± 0.03^{abAB}
8	8.34 ± 0.01^{aB}	8.44 ± 0.02^{abBC}
12	8.42 ± 0.02^{C}	8.49 ± 0.01^{C}

*Means superscripted with different letters in column (^{ABCD}) for a particular data differ significantly from each other ** (P<0.01)*

Rumen Fermentation Parameters

Rumen pH, Total Volatile Fatty Acids and Lactic Acid

In all the treatment groups a significant fall in pH was observed at 4 hr after feeding, possibly due to greater production of volatile fatty acids and lactic acid obtained at similar hour. While at 8 hr post feeding pH tended to increase with a gradual decline in concentration of volatile fatty acids and lactic acid (Table 4). This could be explained on the basis of greater inflow of bicarbonate rich alkaline saliva buffering the ruminal contents. The higher concentration of TVFA and lactic acid at 4 hr after feeding is result of

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stimulated ruminal microbial growth and activity. The results also suggested that supplementation of probiotics causes an elevation of ruminal pH, TVFA with decrease in lactic acid concentration (Kamra *et al.*, 2002; Elseed and Abusamra, 2007; Garg *et al.*, 2009; Thrune *et al.*, 2009; Latif *et al.*, 2014, Khaled and Baraka, 2011). Addition of probiotic mix increased the pH perhaps due to utilization of lactic acid from the ruminal contents, thereby stabilizing pH (Dawson and Tricarico, 2002).

Table 4: Average values of rumen pH, TVFA and lactic acid at different time intervals in treatment groups

Hours	Treatment groups	
	T_0	T_1
Rumen pH		
0	6.84 ± 0.02^{cD}	7.00 ± 0.01^{bD}
2	6.51 ± 0.01^{dB}	6.66 ± 0.02^{cB}
4	6.40 ± 0.02^{bA}	6.56 ± 0.02^{bA}
8	6.52 ± 0.01^{bB}	6.81 ± 0.01^{cC}
12	6.77 ± 0.03^{aC}	6.84 ± 0.03^{bC}
TVFA (mEq/l)		
0	74.44 ± 0.77^{cB}	83.73 ± 0.44^{cB}
2	77.64 ± 0.49^{cC}	87.06 ± 0.37^{bC}
4	93.41 ± 0.53^{aE}	103.50 ± 0.41^{bE}
8	$82.75\pm0.32b^{cD}$	94.38 ± 0.71^{cD}
12	71.87 ± 0.51^{cA}	77.68 ± 0.98^{bA}
Lactic acid (mg/l)		
0	133.68 ± 0.30^{dA}	107.44 ± 0.29^{bA}
2	201.34 ± 0.22^{dD}	173.42 ± 0.30^{bD}
4	249.94 ± 0.55^{dE}	221.82 ± 0.22^{bE}
8	160.58 ± 0.47^{dC}	132.67 ± 0.10^{aC}
12	139.49 ± 0.16^{dB}	112.67 ± 0.18^{aB}

*Means superscripted with different letters in a column (^{ABCD}) for a particular data differ significantly from each other *(P<0.05), ** (P<0.01)*

Nitrogen Fractions

The ammonia nitrogen concentration in rumen was observed at peak level 4 hr post feeding in all the experimental groups. The concentration of ammonia was varying less at 0 hr but showed variation at 4 hr post feeding among the groups. The peak concentration of ammonia at 4 hr was possibly due to maximum proteolytic deaminase activity at this hour, while decrease in concentration at 8h post feeding onwards may be due to simultaneous absorption or its utilization by the microbes in synthetic activity of rumen. Regarding nitrogen and nitrogen fractions *viz*., total nitrogen, TCA-ppt N and NPN also showed similar effect of time of sampling as shown in case of ammonia nitrogen i.e. values increased initially, reached to peak at 4 hr post feeding and then declined continuously up to 12 hr post feeding but there were some variations individually. The peak concentration at 4 hr post feeding might be due to more rumen microbial activity during this period (Table 5). The peak concentration of total-N, NPN, NH_3-N in SRL has also been reported during this period by many workers (Gupta *et al.*, 2006).

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Table 5: Average values of total-N, NH_3-N, TCA-perceptible nitrogen and NPN at different time intervals in treatment groups

Hours	Treatment Groups	
	T_0	T_1
Ammonia Nitrogen (mg/dl)		
0	20.63 ± 0.49^{B}	17.55 ± 0.58^{A}
2	23.85 ± 0.23^{bC}	21.45 ± 0.38^{aB}
4	28.97 ± 0.31^{cD}	25.80 ± 0.37^{bC}
8	23.17 ± 0.36^{bC}	20.66 ± 0.43^{aB}
12	18.02 ± 0.31^{cA}	17.56 ± 0.39^{aA}
Total-N (mg/l)		
0	74.07 ± 1.23^{aA}	98.04 ± 2.47^{bB}
2	101.90 ± 0.34^{aB}	112.37 ± 0.69^{bC}
4	117.54 ± 1.21^{aD}	126.46 ± 0.99^{bE}
8	106.89 ± 0.45^{aC}	117.40 ± 0.88^{bD}
12	73.44 ± 0.90^{aA}	78.89 ± 0.69^{bA}
TCA-Perceptible Nitrogen (mg/dl)		
0	40.80 ± 0.15^{aA}	52.70 ± 1.93^{cB}
2	56.95 ± 0.44^{aB}	67.88 ± 0.47^{bD}
4	62.73 ± 0.78^{aC}	78.14 ± 0.32^{bE}
8	55.47 ± 0.78^{aB}	63.96 ± 0.31^{bC}
12	41.36 ± 0.30^{aA}	49.10 ± 0.49^{bA}
Non Protein Nitrogen (mg/dl)		
0	33.27 ± 1.31^{aA}	45.34 ± 3.22^{bB}
2	44.95 ± 0.68^{aB}	44.49 ± 0.37^{aB}
4	54.81 ± 1.91^{cC}	48.32 ± 1.30^{bB}
8	51.42 ± 1.07^{bcC}	53.44 ± 0.86^{cC}
12	32.08 ± 0.73^{aA}	29.79 ± 0.94^{aA}

*Means superscripted with different letters in a column (^{ABCD}) for a particular data differ significantly from each other *(P<0.05), ** (P<0.01)*

The concentration of ammonia nitrogen in probiotic mix supplemented group decreased significantly compared to control group, may be due to increase the digestion of DM and NDF, more energy substrates are released, improving microbial protein synthesis by reducing the concentration of N-NH_3 (Gado *et al.*, 2011) and increased uptake and assimilation of ammonia nitrogen by rumen microbes due to stimulation of bacterial growth (Garg *et al.*, 2009; Malik and Singh, 2009; Hillal *et al.*, 2011; Comert *et al.*, 2015). Mean values of total nitrogen and TCA ppt.-N was significantly higher in probiotics supplemented group than control. However, for mean value of NPN there was no significant difference observed in feed supplemented groups than control. The higher concentration of total nitrogen and TCA ppt.-N in probiotic mix group might be due to increased utilization of ammonia nitrogen by rumen microbes for microbial protein synthesis. The higher level of total-N may also be attributed to a significantly higher proteolytic activity of the rumen in probiotic mix supplemented groups (Yoon and Stern, 1996).

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Conclusion

In conclusion the counts of rumen microorganisms (total bacteria, *F. succinogenes*, metanogens, fungi and protozoa) were found to be highest at 0 h sampling i.e. before feeding and being least at 2 hour post feeding probably due to dilution effect caused by intake of feed and water by the animals, whereas rumen fermentation parameters like pH was found lowest and total volatile fatty acids, lactic acid and nitrogen fractions concentration highest at 4 hour post feeding.

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